

Title	Natural antimicrobial nanomaterials for food packaging applications
Authors	Sullivan, David Joseph
Publication date	2019
Original Citation	Sullivan, D. J. 2019. Natural antimicrobial nanomaterials for food packaging applications. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2023-05-05 11:31:23
Item downloaded from	http://hdl.handle.net/10468/9471

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College of Science, Engineering and Food Science



UCC

Coláiste na hOllscoile Corcaigh, Éire
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Natural Antimicrobial Nanomaterials for Food Packaging Applications

Presented by

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For the degree of

Doctor of Philosophy in Chemistry

Under the supervision of,

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April 2019

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Declarations

I, David Joseph Sullivan, certify that this thesis is my own research work and I have not obtained a degree in University College Cork or elsewhere on the basis of this PhD thesis.

David J. Sullivan

Acknowledgements

First of all I would like to thanks Prof Michael Morris for the opportunity to undertake this research and for all the help, patience and advice afforded to me. I would also like to thank Prof Joe Kerry for the opportunity. I also wish to thank Dr Malco Cruz-Romero without whose help and advice I would not have made it this far and for continually pushing me to improve on my work.

Secondly I would like to thanks all my lab mates past and present. Being in the unique position of being split between two departments I have made a lot of friends over the years from both chemistry and food science. From the food science side I would like to thank Aislinn, Ciara, Paula, Mike and Shannon for making coming in to work every day so enjoyable and for all the Blas nights out. From the chemistry side, I would like to especially thank; Tim Collins who has continually available for help and advice especially with the AFM and Elsa and Niall who were always ready for a coffee and a chat. In particular, I would also like to thank postdoc's Atul (for showing me the ropes), Cian, Tandra, and Sibú for their help and advice over the years. I would also like to thank everyone past and present Lab 343 and 115 and in the food science group and would also like to thank the technical staff in both chemistry and food science for their help throughout the years. I would also like to take this opportunity to thank Tom O'Mahony for all the help with chapter 5.

Thirdly I would like to thank my friends for their support. The chemistry boys (and girl) of Will, Pa, Eoghan, Tom, Dan Lordan, and Rachel but a special thanks to my housemates Donal (who was always gave good advice) and Dan (for showing me what a good comic is)! Also to my school friends of James, John, Larry, Noel, Ken, and Denis who were always ready for a visit to Cork.

Finally I would like to thank my family, especially my Granda, John Joe, and all my aunts, uncles, and cousins for their support and encouraging questions such as “what is it you actually do?” or “why couldn’t you get a useful job like an electrician”. Most importantly of all, I would like to thank my brother and parents. To my brother, Eoin, who will never let my ego get too big! To my father, John, who would always take the time for a chat despite being incredibly busy himself and for consistently supporting and encouraging me with his interest in what I’m doing and his motto “a change is as good as a rest, now go feed those cows”. To my mother, Catherine, who got me out of bed every morning in my first year of college to make sure I made all my lectures on time! Without their love and support I would never have made it this far and for this I’m forever grateful to them.

“Patience will get a snail to Jericho” – Unknown

Abstract

Consumer demand for fresh-like, sustainable, minimally processed, and clean label food products has been increasing significantly. However, removing chemical preservative and additives from food products significantly reduces their shelf life. To address this challenge, natural antimicrobial nanomaterials can be used to inhibit food spoilage microorganisms and extend the shelf life of food products. This thesis aims to investigate the development of nanomaterials of several of the most effective natural antimicrobial materials including; plant essential oils, antimicrobial polysaccharides, and organic acids and also aims to assess their antimicrobial activity and the application of these natural antimicrobials as coatings either directly or indirectly on food products.

Chapter 1 reviews several of the most effective natural antimicrobials including; plant essential oils, antimicrobial polysaccharides, organic acids and bacteriocins and also aims to review the extrinsic and intrinsic factors that affect their antimicrobial activity, their mode of antimicrobial activity and the application of these natural antimicrobials as coatings either directly or indirectly on food products. Furthermore, this chapter also summarises some commercially available active packaging products and novel surface modification techniques that can be used to develop antimicrobial active packaging systems using natural antimicrobials.

Chapter 2 evaluated the effects of the initial concentrations of chitosan (CS) and sodium tripolyphosphate (TPP), the CS:TPP mass ratio, the CS molecular weight (MW) and pH on the synthesis of CS nanoparticles (CS NPs). The particle size of the synthesised CS NPs was significantly affected ($P < 0.05$) by these parameters. When higher concentrations of CS were used, application of external forces (tip sonication

or Ultra-Turrax™) was necessary to induce monodispersity and significantly ($P < 0.05$) reduce the particle size.

Chapter 3 details the effect of aerosolisation treatments of nano and non-nano natural antimicrobials solutions of chitosan (CS), chitosan nanoparticles (CS NP) and commercially available carnosolic acid nano-solubilise (CASB) as an additional hurdle with vacuum skin packaging (VSP) to extend the shelf-life of highly perishable European Hake (*Merluccius merluccius*) fillets caught off the Irish sea. The microbiological quality of hake fillets aerosolised with natural antimicrobial solutions of CS, CS NP, or CASB, followed by VSP were found to have a significantly ($P < 0.05$) extended shelf-life by up to 40, 50 and 55 %, respectively upon untreated control hake fillets.

Chapter 4 evaluates the effects of different surfactants, the surfactant to oil ratio and physical treatments on the droplet size, polydispersity and zeta potential of oregano nanoemulsions. The antimicrobial activity of monodisperse nanoemulsions were then assessed, followed by application via aerosolisation treatment on highly perishable European Hake (*Merluccius merluccius*) fillets caught off the Irish Sea.

Chapter 5 aims to develop a simple method to attach an antimicrobial essential oil support material to a functionalised surface. SBA-15 was modified with 3-aminopropyltriethoxysilane (APTES) and was then bound to an epoxy modified surface. These materials were loaded with oregano essential oil and their physical properties and antimicrobial activity against food spoilage microorganisms assessed.

Chapter 6 evaluates the antimicrobial effects of benzoic and sorbic acid salt and their nano-solubilises against planktonic and biofilm cultures of *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microflora. The organic acid nano-solubilises

were significantly ($P<0.05$) more effective compared to their organic acid salt counterpart with respect to both planktonic and biofilm cultures. However, biofilms of *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microflora were significantly ($P<0.05$) more resistant to both organic acids salts and nano-solubilisates compared to the planktonic cultures.

Finally, Chapter 7 provides a general overview of the conclusion of each chapter and an outlook to the future of the field.

Thesis Output

Published Publications

- 1) **Sullivan, D. J.**, Cruz-Romero, M., Collins, T., Cummins, E., Kerry, J. P., & Morris, M. A. (2018). Synthesis of monodisperse chitosan nanoparticles. *Food Hydrocolloids*, 83, 355-364.
- 2) **Sullivan, D. J.**, Azlin-Hasim, S., Cruz-Romero, M., Cummins, E., Kerry, J. P., & Morris, M. A. (2018). 11 - Natural Antimicrobial Materials for Use in Food Packaging A2 - Tiwari, Atul. In *Handbook of Antimicrobial Coatings* (pp. 181-233): Elsevier.
- 3) **Sullivan, D. J.**, Azlin-Hasim, S., Cruz-Romero, M., Cummins, E., Kerry, J. P., & Morris, M. A. (2020). Antimicrobial effect of benzoic and sorbic acid salts and nano-solubilisates against *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microbiota biofilms. *Food Control*, 107, 106786.

Submitted for publication

- 1) **Sullivan, D. J.**, Cruz-Romero, M. C., Hernandez, A. B., Cummins, E., Kerry, J. P., & Morris, M. A. Title: Aerosolisation of nano and non-nano natural antimicrobial materials as a novel hurdle with vacuum skin packaging to extend the shelf-life of *Merluccius merluccius* (European hake) fillets. Submitted to *Food Packaging and Shelf life*

In preparation

- 1) **Sullivan, D. J.**, Cruz-Romero, M. C., Cummins, E., Kerry, J. P., & Morris, M. A. Title: Development of monodisperse oregano nanoemulsions for application via aerosolisation as part of a hurdle strategy to extend the shelf life of hake fillets (*Merluccius merluccius*).

- 2) **Sullivan, D. J.**, O'Mahony, T. F., Cruz-Romero, M. C., Cummins, E., Kerry, J. P., & Morris, M. A. Title; Development of a simple method to deliver antimicrobial oil into a food system: possible attachment to food packaging?

Conference posters and presentations

- 3) **Sullivan, D. J.**, Cruz-Romero, M., Collins, T., Cummins, E., Kerry, J. P., & Morris, M. A. Title; Development of size controlled chitosan nanoparticles for antimicrobial food packaging applications. Oral presentation at the European Materials Research Society, 11th – 15th May 2015, Lille, France.
- 4) **Sullivan, D. J.**, Cruz-Romero, M., Collins, T., Cummins, E., Kerry, J. P., & Morris, M. A. Title; Development of size controlled chitosan nanoparticles for antimicrobial food packaging applications. Poster presentation at the 67th Irish Universities Chemistry Research Colloquium, 25th – 26th June 2015, Maynooth, Ireland.

Conference papers

- 1) Hannon, J. C., **Sullivan, D. J.**, Cruz-Romero, M. C., Kerry, J. P., & Morris, M. A. Cummins, E., Title; Migration assessment of chitosan nanoparticles from a spin coated antimicrobial packaging material. 30th European Federation of Food Science and Technology (EFFoST) Conference, 28th – 30th November 2016, Vienna, Austria

Chapter 1: Natural Antimicrobials Materials for use in Food Packaging

David. J. Sullivan, Azlin-Hasim, Malco C. Cruz-Romero, Ana B. Hernandez, Enda Cummins, Joseph P. Kerry, Michael A. Morris

This Chapter was published as a book chapter in “**Sullivan, D.J.**, et al., *11 - Natural Antimicrobial Materials for Use in Food Packaging A2 - Tiwari, Atul*, in *Handbook of Antimicrobial Coatings*. 2018, Elsevier. p. 181-233.”

1.1 Abstract

Consumer demand for fresh-like, sustainable, minimally processed, and clean label food products has been increasing significantly. However, removing chemical preservative and additives from food products significantly reduces their shelf life. To address this challenge, antimicrobial active packaging systems which contain materials within or on the packaging that actively inhibit food spoilage microorganisms can be used to extend the shelf life of food products. This book chapter aims to review several of the most effective natural antimicrobials including: plant essential oils, antimicrobial polysaccharides, organic acids and bacteriocins and also aims to review the extrinsic and intrinsic factors that affect their antimicrobial activity, their mode of antimicrobial activity and the application of these natural antimicrobials as coatings either directly or indirectly on food products. Furthermore, this review will also summarise commercially available active packaging products and novel surface modification techniques that can be used to develop antimicrobial active packaging systems using natural antimicrobials.

1.2 Introduction

Global food security challenges such as an increasing global population (estimated to reach 9 billion by 2050), increasing rural to urban migration, climate change and the waste of packaged food through microbial decomposition and contamination are putting increased pressure on global food production. The Food & Agriculture Organisation (FAO) have estimated that one-third or over 1.3 billion metric tons of all edible food produced for human consumption is either lost or wasted annually throughout the supply chain (FAO, 2011). Given the resource intensive nature of growing, producing, processing, and packaging food products, this loss represent a significant cost to the food industry. Reducing food losses, minimising waste, and

adding-value to food output are highlighted as being ‘of critical importance for humanity and the planet’ in the Sustainable Development Goals (SDGs) adopted under the United Nations’ 2030 Agenda for Sustainable Development (United Nations General Assembly, 2016). Packaging has a significant role in the food supply chain and it is an integral part of both the food processes and the whole food supply chain with the main aim to protect food from contamination. Novel technologies such as antimicrobial active packaging (AAP) containing natural antimicrobials (NAMs) that can extend the shelf life of food products and reduce food wastage are being explored as potential methods to affect these critical issues. The action to reduce food waste and extend the shelf-life of products through the application of AAP could enhance food security and improve sustainability and safety of food products in the food chain.

NAMs are derived from animal, plant and microbial sources and exhibit a broad range of antimicrobial activity against spoilage microorganisms while being characterised by: biocompatibility, biodegradability and low toxicity (Irkin & Esmer, 2015). NAMs are used in food for two main reasons: to control natural spoilage processes (i.e. food preservation), and impede the proliferation of microorganisms (i.e. food safety). The most commonly used commercially available NAMs include organic acids, inorganic materials (although generally not really natural these are included as useful comparators e.g. metal ions such as silver (Ag), gold (Au), copper (Cu) or titanium dioxide (TiO_2)), polysaccharides (e.g. chitosan (CS)), plant derived essential oils and antimicrobials derived from microbiological sources (e.g. nisin). These antimicrobials have been successfully used to develop AAP which involves the coating or incorporation of antimicrobial substances into the packaging material from which they can be released into the food environment inhibiting microbial growth, reduce lipid

oxidation, and extend the shelf life of food products (Kerry, O'Grady, & Hogan, 2006).

NAMs can be used as food ingredients; however, some NAMs such as EO's can affect the organoleptic properties of the food due to their volatile nature; therefore, a system to control the release of these NAMs is required. Surfaces of fresh or processed products are the most likely areas for microbial contamination. Therefore, the controlled release of NAMs from the packaging material in contact with the food product can be more efficient than use of NAMs directly in the foodstuff (Falguera, Quintero, Jimenez, Munoz, & Ibarz, 2011) as the NAM present in the AAP will be released in a controlled manner (Fang, Zhao, Warner, & Johnson, 2017; Gemili, Yemenicioglu, & Altinkaya, 2009).

Increasing consumer demand for sustainable, fresh-like, minimally processed, and clean label food products has driven interest in the use of NAM for food preservation. For the development of AAP, NAMs have been incorporated into packaging materials such as low density polyethylene (LDPE), polyethylene terephthalate (PET) and poly (lactic acid) (PLA) using commercial extrusion or casting methods (Acevedo-Fani, Salvia-Trujillo, Rojas-Grau, & Martin-Belloso, 2015; Albertos, et al., 2015; Antoniou, Liu, Majeed, & Zhong, 2015; Del Nobile, et al., 2009). One of the most widely used packaging materials in the food industry is LDPE due to its' optical transparency, low permeability to water (liquid and vapour) and inert properties. However, the hydrophobicity of LDPE make it problematic to attach NAMs onto the surface of this material. Therefore, surface modifications using chemical or physical treatments are required to increase attachment of the NAMs.

It was reported that NAMs have good antimicrobial activity against Gram-negative and Gram-positive bacteria; however, the antimicrobial activity spectrum of NAMs can be enhanced combining different NAMs (Hu, Wang, Xiao, & Bi, 2015). For example a combination of CS with clove, oregano or eucalyptus EO's have been used for the development of AAP materials with enhanced antimicrobial activity for food packaging applications (Feyzioglu & Tornuk, 2016; S. F. Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013; Paparella, et al., 2016; F. Yang, et al., 2015). Size reduction of NAM has also been shown to enhance the antimicrobial activity compared to their bulk counterparts due to the increased surface area of the NAM (Cushen & Cummins, 2017; Maillard & Hartemann, 2013). The AAP materials containing NAMs have been used in a wide range of foods including: fruits (Dotto, Vieira, & Pinto, 2015), breads (Passarinho, et al., 2014), muscle-based food products such as pork (H. Lu, Shao, Cao, Ou, & Pan, 2016), beef (Vilela, et al., 2016; Vital, et al., 2016) and chicken (Azlin-Hasim, Cruz-Romero, Morris, Cummins, & Kerry, 2015; Chouliara, Karatapanis, Savvaidis, & Kontominas, 2007; Fratianni, et al., 2010), fish (Seyed Fakhreddin Hosseini, Rezaei, Zandi, & Farahmandghavi, 2016; J. Huang, Chen, Qiu, & Li, 2012; Kazemi & Rezaei, 2015) and dairy products such as cheese (O' Callaghan & Kerry, 2016) among others.

To enhance the shelf life of food products through the application of hurdle technology (an intelligent combination of mild treatments), APP have been also used in combination with various mild treatments such as hydrostatic pressure (Gupta, et al., 2012), modified atmospheric atmosphere packaging (MAP) (Rodriguez-Calleja, Cruz-Romero, O'Sullivan, Garcia-Lopez, & Kerry, 2012), vacuum packing (Günlü & Koyun, 2013), and irradiation treatments (Severino, et al., 2015)

This literature review highlights emerging research on the use of NAMs for the development and application of AAP as novel technology to extend the shelf life of food products and reduce food waste.

1.3 Natural Antimicrobials

When NAMs are intended to be used as food preservatives, several factors must be taken into account before application: a detailed knowledge of the NAMs antimicrobial spectrum, the food microflora, intrinsic properties of the food (e.g. pH, water activity, fat and protein content) and its toxicological aspects as the use and consumption of some NAMs have strict use regulations. As low concentrations of NAMs are used on AAP, it is believed that regulatory issues will be less of a concern. It's also important to highlight that AAP can only maintain the initial quality of food products consequently, food products intended to be packaged using AAP systems should be of the highest physical and microbiological quality.

The categories of NAMs that are currently garnering most research include: essential oil extracts, antimicrobial polysaccharides, organic acids and bacteriocins and these NAMs will be discussed in more detail in the following sections.

1.3.1 Essentials Oils and Oleoresins

ISO (2013) define essential oils (EO's) as a product obtained by steam distillation from a natural raw material of plant origin. As these NAMs have a GRAS (Generally Recognised as Safe) status approved by the Food and Drug Administration (FDA) and an acceptability to the consumer attributable to their historical use as a natural flavouring, making them suitable to be used in food contact materials and EO's have the potential to be used on the development of AAP materials for food applications (Burt, 2004; Holley & Patel, 2005; Hyldgaard, Mygind, & Meyer, 2012; Turek &

Stintzing, 2013). Oleoresins and natural extracts obtained through solvent or supercritical fluid extraction instead of steam distillation from a natural raw material of plant origin have also been used as a natural antimicrobial and antioxidant (Rodríguez-Calleja, Cruz-Romero, García-López, & Kerry, 2015; Sahena, et al., 2009).

EO's have a complex composition of low molecular weight aliphatic and aromatic secondary metabolite compounds in which terpenes (such as *p*-cymene, limonene or piene), terpenoids (such as thymol, carvacol or menthol) and phenylpropenes (such as eugenol, cinnamaldehyde or vallinin) are the more predominant compounds (Calo, Crandall, O'Bryan, & Ricke, 2015; Hyldgaard, et al., 2012). The composition of EO's are affected by several extrinsic parameters including the time of harvest, the climate in which they were grown, geographical source and plant genus (Burt, 2004; Calo, et al., 2015; Hyldgaard, et al., 2012). For commercial applications, EO's are standardised by bodies such as the Association Francaise de Normalisation (AFNOR), International Fragrance Association (IFRA), the "Bundesinstitut für Risikobewertung" (BfR), the Research Institute for Fragrance Materials (RIFM), or the Scientific Committee on consumer's Safety (SCCS). A list of commercially available EO's, used for the development of AAP and their antimicrobial activity against microorganisms are listed in Table 1.1.

Due to the thermolabile, photosensitive and volatile characteristic of the EO's, these compounds are susceptible to autoxidation, isomerisation and thermal rearrangements; therefore, EO's must be appropriately stored at low temperatures, protected from light and oxygen sources (Turek, et al., 2013). One of the major drawbacks in the application of EO's as antimicrobials in packaging applications and food products is their hydrophobic character, phase separation with water and strong impact on taste at

higher concentrations (Bilia, et al., 2014; Calo, et al., 2015). To overcome these drawbacks, and get a better integration of these materials into packaging systems, different technologies such as emulsification or encapsulation into support materials have been developed.

1.3.1.1 Emulsification of Essential Oil's

Emulsions are systems defined as two immiscible liquids with one of the liquids being dispersed as spherical droplets within the other, usually in the presence of a surfactant (Figure 1.1) (McClements, 2011; Rao & McClements, 2011). Emulsification of EO's has been shown to improve properties such as stability (McClements, 2011), bioavailability (Walker, Decker, & McClements, 2015), antimicrobial activity (Anwer, et al., 2014), increased surface area (Buranasuksombat, Kwon, Turner, & Bhandari, 2011) and reduce the negative impact on organoleptic properties through controlled release of the volatile constituents of the antimicrobials (Paparella, et al., 2016). Surfactants used for developing emulsions should be food grade; therefore, a limited number of surfactants can be used for food applications. These are broadly classed into several types such as non-ionic (Tweens) and phospholipids (lecithin) many of which are also derived from natural sources.

According to the average droplet size, emulsions are classified as either microemulsions or nanoemulsions. Microemulsions are thermodynamically metastable droplets that have an average diameter over 500 nm and are spontaneously formed upon the addition of a surfactant; however, higher concentrations of surfactant are required to manufacture microemulsions compared to nanoemulsions (Komaiko & McClements, 2016; Tadros, Izquierdo, Esquena, & Solans, 2004). The main destabilisation forces include Ostwald Ripening, which is the integration of smaller droplets into larger droplets, and coalescence, which is where two or more separate

Table 1.1 Antimicrobial activity of commercially available EO's and their applications on food products.

Essential Oil (genus)	Essential oil metabolites with antimicrobial activity	Antimicrobial activity test	Microorganisms Inhibited (MIC value)	Application method for development of AAP systems	Application of AAP systems on food products	References
Basil (<i>Ocimum basilicum</i> L.)	estragol (20 %), linalool (16 %), methyl cinnamic acid (8 %), Methyl cinnamate (8%)	Agar diffusion, MIC	<i>E. coli</i> (12 mg/ml), <i>L. monocytogenes</i> (24 mg/ml), <i>L. innocua</i> (24 mg/ml), <i>S. typhimurium</i> (3 mg/ml)	Dip coating solution, vapourised EO	Cheese	(V. Ghosh, Mukherjee, & Chandrasekaran, 2013; Suppakul, Sonneveld, Bigger, & Miltz, 2008; Teixeira, et al., 2013) (Arancibia, Giménez, López-Caballero, Gómez-Guillén, & Montero, 2014; Hilbig, Ma, Davidson, Weiss, & Zhong, 2016; Hu, et al., 2015; Ma, Zhang, & Zhong, 2016; Ojagh, Rezaei, Razavi, & Hosseini, 2010; Van Haute, Raes, Van der
Cinnamon (<i>Cinnamomum zeylanicum</i>)	(E)-cinnamaldehyde (10.54%), cinnamyl acetate(10.54%). β - phellandrene (3.94%), <i>p</i> -cymene (2.43%)	Spread plating, pour plating, MIC, MBC	<i>L. monocytogenes</i> (8 ppm), <i>S. enteritidis</i> (12 ppm), <i>E.coli</i> (7 ppm)	Marinade, CS- EO film	Shrimp, pork, rainbow trout	

Clove (<i>Syzygium aromaticum</i> L.)	<i>p</i> -eugenol (67.7 %), acetugenol (16.8 %), trans-caryophyllene (10.8 %), humulene (2 %)	Disk Diffusion, MIC	<i>B. subtilis</i> (0.08 w/w%), <i>S. aureus</i> (0.075 w/w%), <i>Proteus vulgaris</i> (0.085 w/w%), <i>P. aeruginosa</i> (0.3 w/w%), <i>Klebsiella pneumonia</i> (0.25 w/w%) <i>S. aureus</i> (0.23 v/v%), <i>E. coli</i> (0.27 v/v%), <i>P. aeruginosa</i> (2 mg/ml), <i>Shigella dysenteriae</i> (11.25 mg/ml), <i>L. monocytogenes</i> (5.625 mg/ml), <i>S. typhi</i> (5.625 mg/ml), <i>Streptococcus pyogenes</i> (1.406 mg/ml) <i>E. coli</i> (5.5 mg/ml), <i>S. typhimurium</i> (2 mg/ml), <i>S. aureus</i> (50	CS-EO film, marinade, dip coating solution, PLA	Grass carp	Meeren, & Sampers, 2016) (Anwer, Jamil, Ibnouf, & Shakeel, 2014; Chaieb, et al., 2007; Teixeira, et al., 2013; Velluti, Sanchis, Ramos, Egido, & Marin, 2003; H. Yang, et al., 2016)
Eucalyptus (<i>Eucalyptus dives</i>)	piperitone (73.5 %), terpinen-4-ol (7.9 %), α -terpinolene (2 %), α -phellandrene (1.9 %)	MBC, MIC,		CS-EO film	Chicken, pork	(Batish, Singh, Kohli, & Kaur, 2008; Delaquis, Stanich, Girard, & Mazza, 2002; Hammer, Carson, & Riley, 1999; H. Lu, et al., 2016; Sharafati Chaleshtori, Taghizadeh, Rafieian-kopaei, & Sharafati-chaleshtori, 2015; Sugumar, et al., 2013)
Garlic (<i>Allium sativum</i> L.)	1(7),5,8-o-menthatriene (20.7 %), di-2-propenyl disulfide	Disk Diffusion, MIC		CS-EO coating,	Pork, shrimp, fresh cut tomato	(Aşik & Candoğan, 2014; Cao, et al., 2013; Pranoto,

	(10.6 %), di-2-propenyltetrasulfide (9.2 %), dimethyl tetrasulphide (4.3 %)		mg/ml), <i>B. cereus</i> (0.08 mg/ml), <i>Salmonella Enteritidis</i> (5.5 mg/ml), <i>L. monocytogenes</i> (0.02 mg/ml)	alginate-EO films		Salokhe, & Rakshit, 2005; Ross, Griffiths, Mittal, & Deeth, 2003; Teixeira, et al., 2013; F. L. Yang, Li, Zhu, & Lei, 2009)
Laurel (<i>Laurus nobilis</i> L.)	linalool (19.97 %), eucalyptol (18.47 %), α -terpinenyl acetate (11.74 %), α -terpineol (4.52 %)	MIC, Disk diffusion	<i>S. aureus</i> (>2000 μ g/ml), <i>E. coli</i> (125 μ g/ml), <i>L. monocytogenes</i> (500 μ g/ml), <i>P. aeruginosa</i> (>2000 μ g/ml)	vapourised EO	Beef	(Baratta, Dorman, Deans, Biondi, & Ruberto, 1998; Munoz, Guevara, Palop, Tabera, & Fernandez, 2009; Ozcan & Erkmén, 2001; C. Ramos, et al., 2012; Yilmaz, Timur, & Aslim, 2013) (Acevedo-Fani, et al., 2015; Falcao, et al., 2012; Hammer, et al., 1999; Moore-Neibel, Gerber, Patel, Friedman, & Ravishankar, 2012; Velluti, et al., 2003)
Lemongrass (<i>Cymbopogon citratus</i>)	geranial (52 %), neral (28 %), limonene (5.4 %), geranyl acetate (3.6 %)	MIC	<i>E. coli</i> (0.06 mg/ml), <i>S. aureus</i> (6 mg/ml), <i>P. aeruginosa</i> (24 mg/ml), <i>Salmonella choleraesuis</i> (24 mg/ml)	Dip coating solution, edible film	Lettuce and spinach inoculated with <i>S. enterica</i> (LAJ160311)	

Oregano (<i>Origanum Vulgare</i>)	carvacol (47.80 %), thymol (21.41%), γ - terpinene (13.44 %), <i>p</i> -cymene (8.53 %)	MIC, Disk diffusion	<i>E.coli</i> (1 mg/ml), <i>L. innocua</i> (2 mg/ml), <i>L. monocytogenes</i> (3 mg/ml), Chicken microflora	Edible films, gelatin-alginate-EO film, marinade, PLA, sachet	Lettuce, rainbow trout, chicken, pork, salmon, shrimp	(Bhargava, Conti, da Rocha, & Zhang, 2015; Javidi, Hosseini, & Rezaei, 2016; Kazemi, et al., 2015; Paparella, et al., 2016; Passarinho, et al., 2014; Teixeira, et al., 2013; Van Haute, et al., 2016; Vital, et al., 2016)
Peppermint (<i>Mentha piperita</i> L.)	menthone (33.34 %), menthol (29.53 %), isomenthone (7.58 %), 1,8-cineol (7 %)	MIC, MBC	<i>Enterococcus faecalis</i> (32 μ l/ml), <i>E.coli</i> (1 μ l/ml), <i>S. aureus</i> (2 μ l/ml), <i>Candida albicans</i> (0.5 μ l/ml)	Pectin films, HDPE	-	(Biddeci, et al., 2016; Edris & Farrag, 2003; Fayaz Dastgerdi, Goli, & Kadivar, 2016; Thosar, Basak, Bahadure, & Rajurkar, 2013)
Rosemary (<i>Rosmarinus officinalis</i> L)	1,8-cineol (26.54 %), α -pinene (20.14 %), camphor (12.88 %), camphene (11.38 %)	MIC, MBC	<i>B. subtilis</i> (0.1 v/v%), <i>E. coli</i> (0.3 v/v%), <i>S. aureus</i> (0.03 v/v%), <i>S. epidermidis</i> (0.1 v/v%), <i>P. aeruginosa</i> (0.1 w/v%)	Edible coating, PLA, gelatin-chitosan	Beef, fish	(Bozin, Mimica-Dukic, Samojlik, & Jovin, 2007; Gomez-Estaca, Lopez de Lacey, Lopez-Caballero, Gomez-Guillen, & Montero, 2010; Y. Jiang, et al.,

Sage (<i>Salvia officinali</i>)	α -thujone (19.9 %), camphor (18.9), viridiflorol (17.5 %), borneol (5.4 %)	MIC, MBC	<i>S. dysentery</i> (8 mg/ml), <i>E. coli</i> (8 mg/ml), <i>S. typhi</i> (32 mg/ml), <i>L. innocua</i> (11 mg/ml), <i>L. monocytogenes</i> (11 mg/ml)	Cellulose paper, edible film, whey protein films	-	2011; Vilela, et al., 2016; Yahyaoui, Gordobil, Diaz, Abderrabba, & Labidi, 2016) (Acevedo-Fani, et al., 2015; Bozin, et al., 2007; Moghimi, Aliahmadi, McClements, & Rafati, 2016; Royo, Fernandez-Pan, & Mate, 2010; Teixeira, et al., 2013) (Acevedo-Fani, et al., 2015; Fratianni, et al., 2010; Gomez-Estaca, et al., 2010; Gutierrez, Barry-Ryan, & Bourke, 2009; Kazemi, et al., 2015; Van Haute, et al., 2016; Yahyaoui, et al., 2016)
Thyme (<i>Thymus zygis</i>)	thymol (55.91%), <i>p</i> -cymene (20.61%), γ -terpinene (5.59%), linalool (3.30%)	MIC, MBC	<i>Enterococcus fecalis</i> (32 μ l/ml), <i>E. coli</i> (2 μ l/ml), <i>S. aureus</i> (32 μ l/ml), Salmon microflora, Chicken microflora	CS - EO film, dip coating solution, PLA, marinade	Salmon, chicken, pork, rainbow trout	

droplets come together forming a larger droplet (Komaiko, et al., 2016; McClements, 2011; Walker, et al., 2015).

Nanoemulsions are kinetically metastable droplets that have an average diameter < 500 nm; however, these are non-spontaneous systems requiring size reducing treatments using high energy or low energy techniques in order to decrease the overall droplet diameter (McClements, 2011). Nanoemulsions are less susceptible to the effects of gravitational force and sedimentation than microemulsions as a consequence of greater Brownian motion and diffusion rate (Tadros, et al., 2004). Destabilisation forces can be overcome by the inclusion of a co-surfactant which acts synergistically to avoid aggregation of nanoemulsions (Komaiko, et al., 2016; Tadros, et al., 2004).

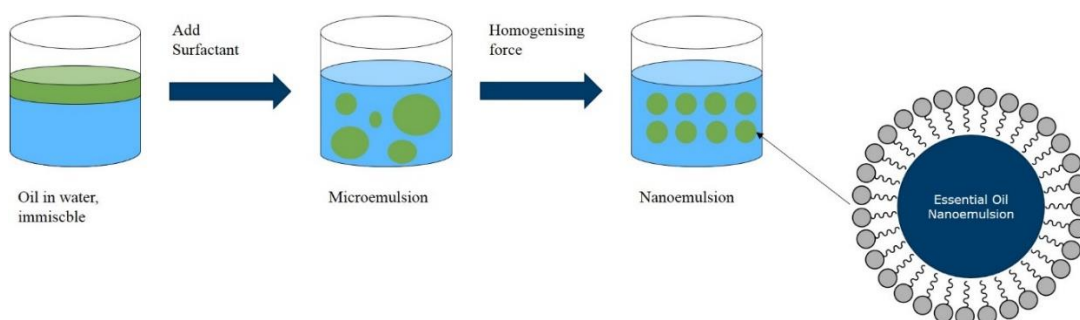


Figure 1.1 Schematic of nanoemulsion formation. Due to the immiscibility of oil and water, surfactants are added to disperse the hydrophobic oil in water. By using homogenisation forces such as tip sonicators etc. the overall droplet diameter of the emulsion can be reduced resulting in the formation of metastable nanoemulsions.

1.3.1.1.1 High Energy Techniques

High energy techniques use mechanical force to reduce the overall droplet size, where the required energy for size reduction is determined by the Laplace pressure (ΔP) (Eq. 1.1) which increases with decreasing droplet size, where r is the droplet radius and γ is the interfacial tension of the droplet.

$$\Delta P = \frac{2\gamma}{r} \quad (1.1)$$

The force required to develop nanoemulsions will be dependent on the composition factors such as the physicochemical properties of the oils, the emulsifier and their relative concentrations to each other. Moreover, physicochemical properties of developed nanoemulsions such as interfacial tension and viscosity will also influence the amount of energy needed to form nanoemulsions. Herein two of the primary techniques used to develop food grade nanoemulsions are high pressure homogenisation and high intensity ultrasound are discussed below.

1.3.1.1.1 High Pressure Homogenisation of Essential Oil Emulsions

The principal type of homogenising force used is high pressure homogenisation (HPH) which operates by feeding a coarse O/W emulsion solution through an outlet valve at high pressures of 200 – 300 MPa in a confined chamber to generate intense shear forces and turbulence that reduce overall droplet diameters of O/W emulsions and can result in nanoemulsions (Figure 1.2) (Donsì & Ferrari, 2016).

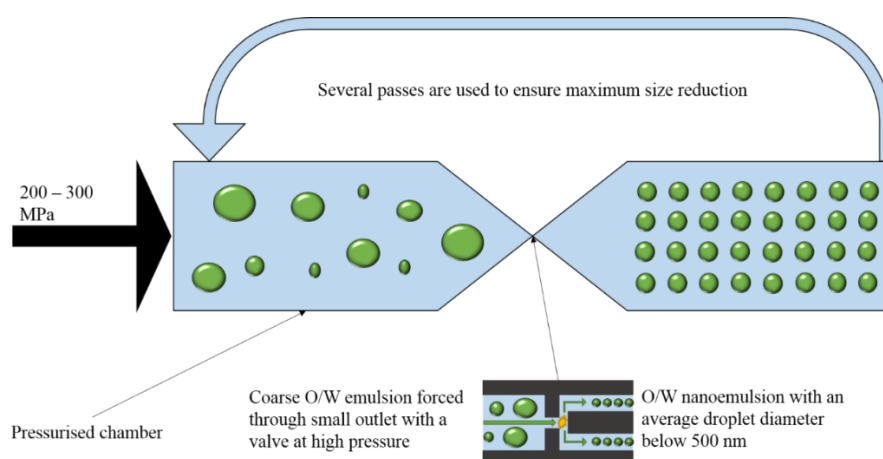


Figure 1.2 Schematic of high pressure homogenisation of essential oils nanoemulsions

1.3.1.1.2 Sonication of Essential Oil Emulsions

High energy sonication with a frequency greater than 20 Hz has also been demonstrated to create nanoemulsions through an acoustic energy (Figure 1.3) (V. Ghosh, et al., 2013). This technique uses acoustic energy to create rapidly collapsing bubbles in solution which generates a high pressure gradient and high velocity in the liquid creating a shear force that will break up the coarse O/W emulsion into a nanoemulsion (V. Ghosh, et al., 2013).

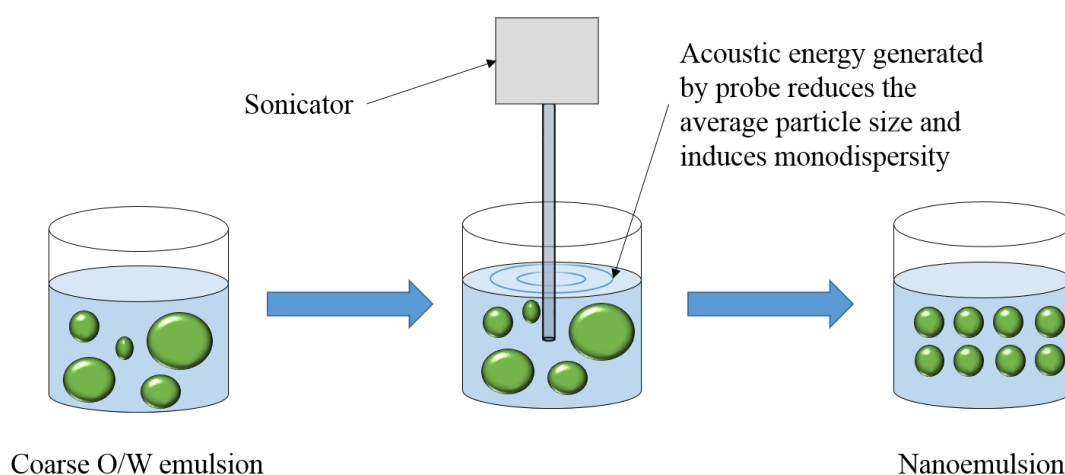


Figure 1.3 Schematic of high pressure homogenisation of essential oils nanoemulsions.

1.3.1.1.2 Low Energy Techniques

More recently, low energy techniques have been investigated such as Phase Inversion Temperature (PIT) or Emulsion Inversion Point (EIP). These techniques primarily use the manipulation of composition and temperature of an emulsion to change the surfactants physicochemical properties. These methods are seen as more commercially favourable as they require less energy to form to form an emulsion; however, are generally limited to use with small non-ionic surfactants (Komaiko, et al., 2016).

1.3.1.1.2.1 Phase Inversion Temperature formation of Essential Oil Nanoemulsions

Phase inversion temperature (PIT) uses the change in solubility of non-ionic surfactants as a result of changes in temperature to generate nanoemulsions (Figure 1.4) (Moraes-Lovison, et al., 2017). At low temperature, the hydrophilic headgroup is fully hydrated; however, as temperature increases this hydrophilic headgroup becomes dehydrated and more hydrophobic resulting in a change of emulsion phase from O/W to W/O at which point rapid cooling results in ultrafine nanoemulsions. This technique takes advantage of the reduced interfacial tension at higher temperature which forms an intermediate bicontinuous layer and when rapidly cooled the change in interfacial tension coupled with the turbulent flow results in nanoemulsions (Rao & McClements, 2010; Walker, et al., 2015).

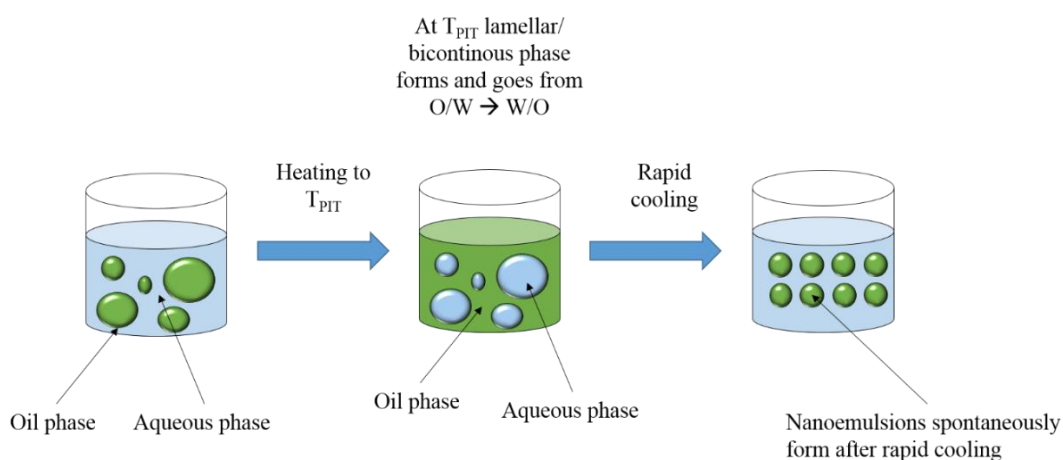


Figure 1.4 Schematic of phase inversion temperature (PIT) formation of nanoemulsions

1.3.1.1.2.2 Emulsion Inversion Point formation of Essential Oil Nanoemulsions

In this method, the surfactant and oil are added first, followed by the gradual addition of the aqueous phase over time and under constant agitation (Figure 1.5). The gradual addition of water results in the W/O emulsion turning into an O/W emulsion at a

critical catastrophic phase inversion point whereupon nanoemulsions spontaneously form. The average droplet diameter will be dependent on the agitation speed and rate of water addition to the W/O system. A substantial drawback to this type of synthesis is that it only works with small molecule non-ionic surfactants (Komaiko, et al., 2016).

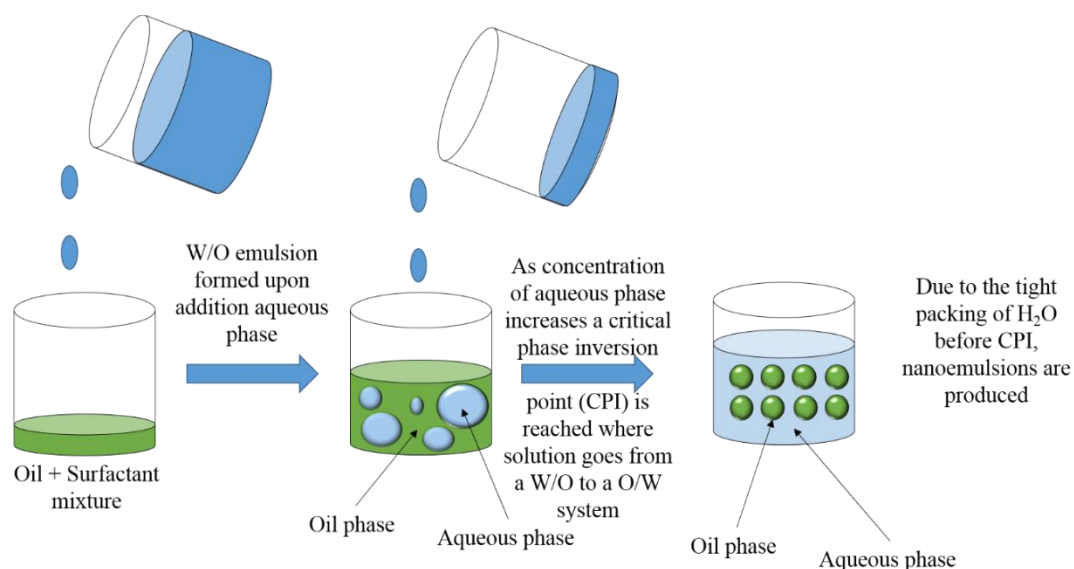


Figure 1.5 Schematic of catastrophic inversion point (CPI) formation of nanoemulsions

1.3.1.2 Encapsulation of Essential Oils into a Support Material

Another approach is the encapsulation of EO's into a support porous siliceous material such as Santa Barbara Amorphous (SBA)-15 or Mobil Composition of Matter No. 41 (MCM-41) (Sun, Lu, Qiu, & Tang, 2017). These materials have been used to protect EO's from environmental stressors while also reducing their negative organoleptic impact and interaction with food components and allowing for a controlled release of the EO (Ruiz-Rico, et al., 2017). In particular, research has been carried out on the encapsulation of EO materials into SBA-15 due to these materials current use in the food sector as catalysts in the synthesis of nutrients, bioactive molecules, in sensor technology and as carriers to design smart delivery systems (Ruiz-Rico, et al., 2017). Moreover, these materials have mechanical and hydrothermal stability (Hernández-

Morales, et al., 2012), adjustable nano-pore size (Sun, et al., 2017), ordered pore structure (Maria Chong & Zhao, 2003), large specific surface area ($\sim 1000 \text{ m}^2 \text{ g}^{-1}$) (Maria Chong, et al., 2003) and relatively large void volume (Hernández-Morales, et al., 2012) and are also considered GRAS and are an authorised additive in the European Union under E-551 (Ruiz-Rico, et al., 2017).

SBA-15 materials can readily undergo functionalisation with various organic functional containing groups such as 3-aminopropyltriethoxysilane (APTES) which are covalently linked on the wall of the porous structure via hydrolysis and/or condensation reactions giving rise to an amine modified porous silica while retaining the mesoporous silica's favourable physical properties (Vilarrasa-García, et al., 2014).

1.3.1.3 Factors that affect Antimicrobial Activity of Essential Oils

In bacteria, the Gram stain provides an important classification system, as several cell properties can be correlated with the cell envelope affecting the antimicrobial activity of the EO's (Mai-Prochnow, Clauson, Hong, & Murphy, 2016). Gram-positive cell membranes have a thick rigid 3D structured peptidoglycan layer (around 20-80 nm) formed by the cross-linking of the short peptides and linear polysaccharides of the cell wall. In contrast, Gram-negative bacteria cell membranes are chemically and structurally more complex, however, they possess thinner layers of peptidoglycan ($< 10 \text{ nm}$), but harbour an additional outer membrane with several pores and appendices (Hajipour, et al., 2012; Mai-Prochnow, et al., 2016). These differences in the cell envelope confer different properties to the cell, in particular responses to external stresses, including heat, UV radiation and antibiotics (Mai-Prochnow, et al., 2016).

Table 1.2 Methods used to develop essential oil nanoemulsions for food applications

Essential/extract oil	Food-grade surfactant(s) and/or encapsulator(s) used	Method of nanoemulsification	Average droplet diameter (nm)	Antimicrobial activity against food products/food-borne pathogens	Ref
Anise	medium-chain triacylglycerol, soy lecithin	HPH	117 - 276	<i>Listeria monocytogenes</i> , <i>E.coli</i> O157:H7	(Topuz, et al., 2016)
Basil	Tween 80	High energy ultrasound	29 - 41	<i>E. coli</i>	(V. Ghosh, et al., 2013)
Bergamont	Tween 20, glycerol monooleate	HPH	163	Green Beans, <i>E. coli</i> , <i>Salmonella typhimurium</i>	(Severino, et al., 2015)
Carvacol	Tween 20, Tween 40, Tween 60, Tween 80, Tween 85, glycerol monooleate, CS NPs	HPH, spontaneous emulsification, ionic gelation	133, 55 – 1300, 40 -80	Green Beans, <i>E. coli</i> , <i>Salmonella typhimurium</i> , <i>Staphylococcus aureus</i> , <i>Bacillus cereus</i>	(Y. Chang, McLandsborough, & McClements; Keawchaoon & Yoksan; Severino, et al., 2015)

Cinnamon	alcohol polyoxyethylene ether, CS NPs	Ionic gelation	527	Pork	(Hu, et al., 2015)
Cinnamon Bark	Tween 20, Tween 80, Lauric arginate	Ultra turrax	100	<i>Salmonella enteritidis</i> , <i>E. coli</i> O157:H7, <i>Listeria monocytogenes</i> , 2 % reduced fat milk (@ 4 °C)	(Hilbig, et al., 2016)
Citral	Span 85, Brij 97	High energy ultrasound	28 – 410	<i>Staphylococcus aureus</i> , <i>E. coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Enterococcus faecalis</i> , <i>Salmonella typhimurium</i> , <i>Listeria monocytogenes</i>	(W.-C. Lu, et al., 2017)
D – Limonene	Soy lecithin, Tween 20, glycerol monooleate	HPH	130	Pear and orange juice, <i>E. coli</i> , <i>Lactobacillus delbrueckii</i>	(Donsì, Annunziata, Sessa, & Ferrari, 2011)
Eucalyptus	Tween 80, Polyoxyethylene (20) sorbitan monolaurate	High energy ultrasound	17.1	<i>Bacillus cereus</i> , <i>E. coli</i> , <i>Staphylococcus aureus</i>	(Sugumar, et al., 2013)
Eugenol	Tween 20, Tween80	High energy ultrasound	13	Fruit juice, <i>Staphylococcus aureus</i>	(Vijayalakshmi Ghosh, Mukherjee, & Chandrasekaran, 2014)

Ginger	Tween 80	High energy ultrasound	57	Chicken breast fillets, <i>Listeria monocytogenes</i> , <i>Salmonella typhimurium</i>	(Noori, Zeynali, & Almasi, 2018)
Lemon	Tween 20, glycerol monooleate	HPH	176	Green Beans, <i>E. coli</i> , <i>Salmonella typhimurium</i>	(Severino, et al., 2015)
Lemongrass	Tween 80	Microfluidiser, high shear force homogeniser	41	<i>E. coli</i> O157:H7, <i>Salmonella typhimurium</i> , Fuji apples, grapes	(Acevedo-Fani, et al., 2015; Oh, et al., 2017; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015)
Mandarin	Tween 20, glycerol monooleate	HPH	161	Green Beans, <i>E. coli</i> , <i>Salmonella typhimurium</i>	(Severino, et al., 2015)
Oregano	Tween 80, Span 80, PEG-40 hydroxylated castor oil	High energy ultrasound, PIT	148, 50	<i>Listeria monocytogenes</i> , <i>Salmonella typhimurium</i> , <i>E. coli</i> O157:H7, Romaine and Iceberg lettuce, chicken pâté, low fat cut cheese	(Artiga-Artigas, Acevedo-Fani, & Martín-Belloso, 2017; Bhargava, et al., 2015; Moraes-Lovison, et al., 2017)
Peppermint	Purity Gum	High speed homogeniser	184 – 228	<i>Listeria monocytogenes</i> , <i>Staphylococcus aureus</i>	(Liang, et al., 2012)

Sage	Tween 80, Span 80	Microfluidiser, High energy ultrasound	35, 222	<i>E. coli, Shigella dysentery, Salmonella typhi</i>	(Acevedo-Fani, et al., 2015; Moghimi, et al.)
Soybean	Triton X -100	Microfluidiser	308	<i>Streptococcus mutans, Lactobacillus casei</i>	(Ramalingam, Amaechi, Ralph, & Lee, 2012)
Summer savory	Tween 80, CS NPs	Magnetic stirring & ionic gelation with CS NPs	135 – 237	<i>E. coli, Staphylococcus aureus, Listeria monocytogenes</i>	(Feyzioglu, et al., 2016)
Terpene mix (from <i>Melaleuca alternifolia</i>)	Soy lecithin, Tween 20, glycerol monooleate	HPH	75	Pear and orange juice, <i>E. coli</i> , <i>Lactobacillus delbrueckii</i>	(Donsi, et al., 2011)
Thyme	Tween 80	Microfluidiser	82	<i>E. coli</i>	(Acevedo-Fani, et al., 2015)

It is widely reported that EO's are more effective against Gram-positive bacteria due to the susceptibility of their lipophilic ends on lipoteichoic acid in the cell membrane facilitating the penetration of hydrophobic EO's whereas the reduced susceptibility seen in Gram-negative bacteria can be attributed to the role of extrinsic membrane proteins and cell wall lipopolysaccharides limiting the diffusion of hydrophobic compounds into the microorganism (Hylgaard, et al., 2012; Tongnuanchan & Benjakul, 2014). While oregano and clove EO's have been shown to exhibit good antimicrobial activity against both Gram-positive and Gram-negative bacteria; EO's of sage, rosemary and cumin have shown antimicrobial activity against Gram-positive bacteria only (Holley, et al., 2005). Additionally, the shape of the bacteria has been also reported to affect the antimicrobial efficacy of EO's, where rod shaped cell have been reported to be more sensitive than coccoid shaped cells (Cui, Zhao, & Lin, 2015; Hajlaoui, et al., 2009).

Food-grade nanoencapsulators and nanoemulsions have been developed as several studies have shown nano-emulsification of EO's can increase antimicrobial activity compared to non-nanoemulsified EO's. Donsì, et al., (2011) developed nanoemulsions and nanocapsules of D-limonene and a mixture of terpenes (from *Melaleuca alternifolia*) using either soy lecithin, Tween® 20 or glycerol monooleate as emulsifiers. To reduce the average droplet diameter, emulsions were high pressure homogenised at 300 MPa and obtained particle sizes between 75 – 175 nm. When these nanoemulsions were applied to pear or orange juice inoculated with *Lactobacillus delbrueckii* (*L. delbrueckii*) the terpene based nanoemulsions delayed the growth of *L. delbrueckii* by 5 days in orange juice and by 2 days in pear juice compared to untreated juices. Anwer, et al. (2014) also reported an enhanced antimicrobial activity of clove EO nanoemulsions with an average particle diameter

of 29.1 nm against *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*), *Proteus vulgaris*, *Pseudomonas Aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumoniae* compared to non-emulsified clove EO. Conversely, some studies reported that nanoemulsions do not enhance the antimicrobial properties of EO's. For example, Buranasuksombat, et al. (2011) reported that nanoemulsions of lemon myrtle oil and soybean oil showed no greater activity over their natural oil counterparts and suggesting that the mode of action of EO's was due to their active components as opposed to the increased surface tension from being in a nanoemulsion state.

Studies have shown that most food grade surfactants do not have an inherent antimicrobial activity; however, their physicochemical properties such as hydrophilic-lipophilic balance (HLB) and polarity affect the oil and surfactants interactions and in course affecting the release of the metabolite present in the oil (Martins, Rodrigues, Barreiro, & Rodrigues, 2011).

The pH of the food matrix's environment will also affect the release of active compound of the EO nanoemulsions. For instance, if the environment is too acidic, the nanoemulsion will destabilise, losing its' homogenous distribution (Fathi, Mozafari, & Mohebbi, 2012). As outlined previously, conflicting results are reported in the literature; however, most studies reported increased antimicrobial activity of the EO's nanoemulsions which might be affected by the active metabolite composition of the EO's and interaction of the metabolites with the surfactant and EO.

1.3.1.4 Mode of Antimicrobial Action of Essential Oils

The antimicrobial mechanism of action of EO's is not fully understood but is reported to be from a synergistic action of all the secondary metabolites such as p-cymene,

thymol, carvacol, eugenol and cinnamaldehydes commonly present in clove, oregano, rosemary, thyme, sage, and vanillin EO's (Figure 1.6) (Hyldgaard, et al., 2012)

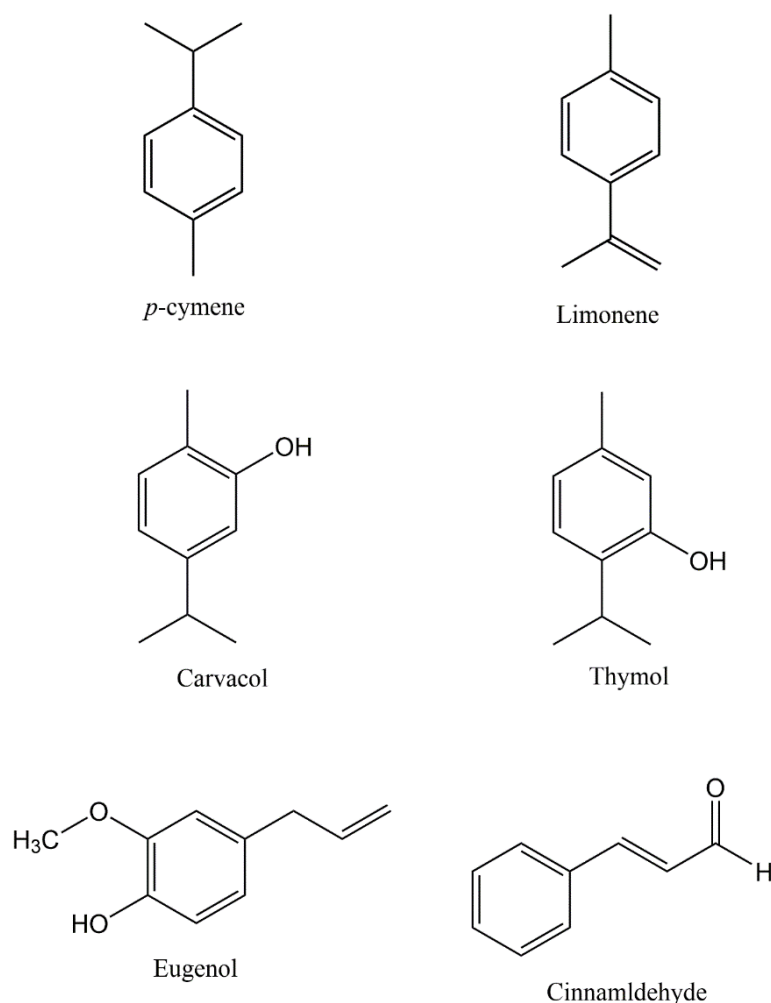


Figure 1.6 Common secondary metabolite terpenes, terpenoids and phenylpropanoids found in essential oils

- i. P-cymene is one of the main terpenes found in EO's, particularly in thyme and oregano. While p-cymene is not inherently antimicrobial, it does have a high affinity for bacterial cell membranes where it can substitute itself into the cell membrane altering the physiological barrier properties facilitating easier access for other more potent antimicrobial compounds (Burt, 2004).
- ii. Terpenoids such as thymol and carvacrol can affect the cellular membrane allowing for the passive transport of ions through the membrane. Carvacrol in particular can disintegrate outer membranes of Gram-negative bacteria while in

Gram-positive bacteria the membrane permeability is altered allowing permeation cations like H^+ and K^+ (Hyldgaard, et al., 2012).

- iii. Phenylpropanoids such as eugenol and cinnamaldehyde can non-specifically permeabilise the cell membrane and can crosslink covalently with internal DNA and amino groups of proteins, respectively (Hyldgaard, et al., 2012; Rodríguez-Calleja, et al., 2015; Tongnuanchan, et al., 2014).

While these are the primary metabolites which impart the EO's antimicrobial activity, other antimicrobially active constituents are covered in detail by Hyldgaard, et al. (2012) or Burt, (2004).

1.3.1.5 Applications of Essential Oil's in Food Packaging Systems

Careful consideration on the natural characteristics of the EO's must be taken when developing EO based APP systems, as their volatile, thermolabile nature does not make them an ideal NAM for use in manufacturing AAP using thermal based processing techniques such as extrusion. Therefore, EO based AAP systems have been developed impregnating these NAM in sachets, dip coatings and edible films.

The volatile nature of the EO's make them ideal substances for their incorporation into sachets which are bonded to the packaging material used and release the volatile antimicrobial compound into the packaging headspace (Otoni, Espitia, Avena-Bustillos, & McHugh, 2016) potentially also reducing the negative impact that EO's can have on the organoleptic properties of food products (Dainelli, Gontard, Spyropoulos, Zondervan-van den Beuken, & Tobback, 2008). An example on the application of this technology is reported by Ayala-Zavala & González-Aguilar, (2010) which encapsulated garlic essential oil into β -cyclodextrin capsules and placed them in cellulose tea bags and used to extend the shelf life freshly cut tomatoes. These

authors reported a significant reduction in microbial spoilage of freshly cut tomatoes compared to controls without garlic EO. Sachets and pads containing EO's have also been used to preserve breads (Passarinho, et al., 2014), papaya (Espitia, et al., 2011) and mangoes (Medeiros, Soares, Polito, De Sousa, & Silva, 2011), among others.

To enhance both flavour and antimicrobial activity, EO's can also be used for dip coating directly onto food products. For instance, Moore-Neibel, et al. (2012) dip coated romaine lettuce, iceberg lettuce, spinach and baby spinach inoculated with *Salmonella enterica* (*S. enterica*) ($6 \log_{10}$ cfu/ml) into saline solutions of lemongrass EO. Lemongrass EO coating reduced up to $4.3 \log_{10}$ cfu/ml of *S. enterica* on iceberg lettuce while reductions on baby spinach, mature spinach and romaine lettuce were lower. Lu, et al. (2016) immersed fresh pork in a solution containing 4 v/v% eucalyptus EO and 1 v/v% Tween 80 for 30 s. It was observed that after 10 days storage under refrigeration (4°C) *P. aeruginosa* populations were significantly lower compared to untreated samples without eucalyptus EO. Oregano, cinnamon and thyme EO's have also been used in marinades formulations containing water, Na-lactate/lactic acid buffer (2 w/w %), NaCl (10 w/w %), and EO emulsified with Tween 80 and applied on meats (pork filet, pork bacon, chicken filets, chicken skin), salmon and scampi. The effects of the marination process had a twofold effect of adding flavour and extending shelf life (Van Haute, et al., 2016).

However, applications of EO's on food products is somewhat limited due to the EO's characteristics such as strong hydrophobicity and volatility which may cause off-flavours and odours within the packaged product. Therefore, techniques such as incorporation into active edible films, carriers or encapsulation, could offer controlled release of EO's into the food environment reducing the impact on organoleptic properties while delivering the antimicrobial properties of the EO's more effectively.

Volpe, et al. (2015) used carrageenan-essential lemon oil active edible coatings on trout fillets and reported that the formed protective thin film limited microbial spoilage to $5.7 \log_{10} \text{ cfu/g}$ after 15 days storage compared to uncoated (without lemon EO) trout fillets which had a TVC of $12.09 \log \text{ CFU g}^{-1}$. Gomez-Estaca, et al. (2010) developed gelatin-chitosan films incorporated with clove essential oil. The clove-containing films inhibited *Pseudomonas fluorescens* (*P. fluorescens*), *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Listeria innocua* (*L. innocua*), *Escherichia coli* (*E.coli*) and *Lactobacillus acidophilus*. The gelatin-chitosan film incorporated with clove essential oil was applied to fish and the growth of Gram-negative bacteria was drastically reduced, especially *enterobacteria*, however, lactic acid bacteria were not affected during chilled storage. The application of chitosan-gelatin incorporated with clove film on fish delayed or even prevented both the growth of microorganisms and the occurrence of total volatile nitrogen indicating the viability of these films for fish preservation extending the shelf-life of fish stored at chilling conditions. Ojagh, et al. (2010) developed an active CS + cinnamon EO coating to be utilised as a safe preservative for fish under refrigerated storage. Successful inhibition of lipid oxidation and microbial growth in refrigerated rainbow trout fillet coated with CS + cinnamon EO which also kept the sensory characteristics within acceptable limits throughout storage. CS + cinnamon EO treatments maintained trout fillet shelf life until the end of the storage period (day 16) without any significant loss of texture, odour, colour or overall acceptability and without significant microbial growth, while control samples had a shelf life of only 12 days.

Nanoemulsions of EO's have also shown antimicrobial activity against food borne bacteria such as *S. aureus*, *Bacillus cereus* (*B. cereus*), *E. coli*, *Shigella dysentery*, *Salmonella typhi* (*S. typhi*) (Moghimi, et al., 2016; Sugumar, et al., 2013). Bhargava,

et al. (2015) applied oregano nanoemulsion to lettuce and observed that there were a significant reduction in concentration of *Listeria monocytogenes* (*L. monocytogenes*), *Salmonella typhimurium* (*S. typhimurium*) and *E. coli* (O157:H7) compared to uncoated control samples. Hilbig, et al. (2016) developed cinnamon bark oil nanoemulsion made with a co-emulsion of Tween 80 and lauric arginate and applied it to 2 % reduced fat milk. The results showed a significant reduction of spoilage and pathogenic bacteria such as *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella enteritidis*.

EO's loaded into mesoporous silica supports for EO delivery in food applications have been reported by authors such as Park, Barton, & Pendleton, (2011) who loaded natural antimicrobial allyl isothiocyanate into MCM-41 and SBA-15 and found they were antimicrobially active against *Escherichia coli*, *Bacillus cereus*, and *Pichia anomala*. Ruiz-Rico, et al. (2017) tested the antimicrobial activity of vanillin grafted onto the surface of MCM-41 and reported that a significant ($P < 0.05$) reduction of 1.5 log CFU mL⁻¹ in the concentration of *Listeria innocua* in pasteurized skimmed milk.

Natural EO's have also shown a limited potential for developing AAP manufactured using conventional commercial polymers such as PLA, polypropylene (PP) and LDPE due to the thermal sensitivity of the EO's. Javidi, et al. (2016) developed AAP of PLA containing oregano EO and used this material to wrap rainbow trout. After 12 days chilled storage, rainbow trout samples wrapped with PLA – oregano EO films were still acceptable for consumption as the microbiological bacterial load threshold of 7 log₁₀ cfu/ml for the safe cooking of raw fish was not reached whereas control rainbow trout samples wrapped with PLA films without oregano EO were above this threshold. Cinnamon, oregano and clove EO's have also been applied onto PP and polyethylene/ethylene vinyl alcohol copolymer and tested against pure culture of *S.*

aureus, *B. cereus*, *Enterococcus faecalis*, and *L. monocytogenes*, *E. coli*, *Yersinia enterocolitica*, *Salmonella choleraesuis*, and *P. aeruginosa*, suggesting good potential for extending shelf life of food products (Lopez, Sanchez, Batlle, & Nerin, 2007).

AAP materials have been used in combination with other mild preservation technologies to improve the safety and shelf life of food products. Chouliara, et al. (2007) used oregano EO and MAP (30/70 % CO₂/N₂) to pack chicken fillets and were stored at 4 °C. It was observed that the use of oregano EO in conjunction with MAP and chilled storage at 4°C doubled the shelf life of chicken fillets compared to samples stored aerobically. Severino, et al. (2015) used EO nanoemulsion, chitosan, gamma radiation and MAP (60 % O₂, 30 % CO₂ and 10 % N₂) treatments as hurdles to extend the safety and shelf life of green beans and that the application of hurdle technology showed a significant reduction in populations of *E. coli* and *S. typhimurium* compared to control (untreated) green beans.

1.3.2 Antimicrobial Polysaccharides

Polysaccharides such as hyaluronic acid, alginates and pectin's are used as film forming compounds; however, chitosan, the deacetylated derivate of chitin, is of particular interest as it has a wide spectrum antimicrobial activity (Moreira Mdel, Pereda, Marcovich, & Roura, 2011)

1.3.2.1 Chitosan

Chitin is the second most abundant polysaccharide found naturally after cellulose and is extracted from fungi, crustaceans and arthropods shells, with the majority of commercial chitin originating from waste shrimp shells, a by-product from the seafood industry (Hamed, Özogul, & Regenstein, 2016). Chitin is poorly soluble in water, therefore, to improve its' water solubility, chitin can undergo chemical modifications

such as esterification or more importantly deacetylation which can be used to form chitosan (CS) (Pillai, Paul, & Sharma, 2009). Deacetylation removes the acetyl group from the acetyl amine on the C-2 position of the glucosamine unit through a sodium hydroxide (NaOH) wash, which can then protonate the anionic amine ($-\text{NH}^-$) to form an amine ($-\text{NH}_2$) group. This amine group can undergo further protonation (to $-\text{NH}_3^+$) in acidic aqueous solution to facilitate CS dissolution and enhance antimicrobial activity (Kong, Chen, Xing, & Park, 2010). CS has been identified to have numerous favourable properties for applications in the food industry (Table 1.2). These properties are biodegradability, biocompatibility, anti-microbial properties along with its' relative abundance and cheap cost (Kong, et al., 2010; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Rampino, Borgogna, Blasi, Bellich, & Cesaro, 2013; Ryan, et al., 2016). Structurally CS is made up of a random assortment of $\beta(1-4)\text{N}$ -acetyl-D-glucosamine linkages where the ratio of the D-glucosamine to N-acetyl-D-glucosamine affects the viscosity and self-aggregating behaviour properties of bulk chitosan (H. K. S. Souza, Campiña, Sousa, Silva, & Gonçalves, 2013).

Due to its physicochemical properties such as film forming ability, non-toxicity, good gas, aroma and lipid barrier properties in addition to antimicrobial activity, CS has widely used to develop edible films (Aloui & Khwaldia, 2016). CS comprises of monosaccharides moieties bonded through glycosidic linkages which contain an abundance of hydroxyl ($-\text{OH}$) functional group's which influence the solubility and structural conformation of films through hydrogen bonding allowing for the formation of edible coatings. CS can also be used as thickening, gelling or hydrating agent. Mechanical and barrier properties of CS films are affected by molecular weight and degree of deacetylation (DD). Higher molecular weights and viscosities form films

with higher tensile strength whereas higher DD of CS decreased the elongation of break and reduced the moisture vapour permeability (Aloui, et al., 2016).

To improve water solubility of CS, CS oligosaccharides and CS quaternary ammonium salts were developed through modification of the amino group on CS via depolymerisation of CS with either enzymes (such as chitosanase, pepsin) or through chemical modifications (such depolymerisation with acids like HCl) increasing water solubilisation of CS at neutral pH. However, studies reported that these modifications negatively affected the antimicrobial activity against *E. coli* and *S. aureus*, compared to native CS (Marini, Bondi, Iseppi, Toselli, & Pilati, 2007; Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2009). These chemically modified CS have been applied on products such as Kimchi (No, Meyers, Prinyawiwatkul, & Xu, 2007) and on sweet cherries (Feliziani, Santini, Landi, & Romanazzi, 2013) among others.

1.3.2.2 Chitosan Nanoparticles

Chitosan, a deacetylated form of chitin, the second most abundant polysaccharide found naturally after cellulose and is extracted from fungi, crustaceans and arthropod shells (Hamed, et al., 2016). The structure of chitosan consisting of a random assortment of $\beta(1-4)$ N-acetyl-D-glucosamine linkages contribute to its unique properties such as acid solubility and broad spectrum antimicrobial activity (Figure 1.7). The primary proposed modes of antimicrobial activity is from: electrostatic interactions with negatively charged components of bacterial cell membranes (Pilon, et al., 2015), bind to DNA and inhibit RNA replication (Cruz-Romero, Murphy, Morris, Cummins, & Kerry, 2013b), form a thin layer film over the bacterial cell inhibiting the absorption of nutrients (S. H. Chang, Lin, Wu, & Tsai, 2015) and are covered in detail by Sullivan, et al. (2018). The potential applications of CS NPs in active packaging are being extensively investigated and have been used as edible

coatings and films (Farajzadeh, Motamedzadegan, Shahidi, & Hamzeh, 2016; Y. B. Wang, et al., 2015; Zarei, Ramezani, Ein-Tavasoly, & Chadorbaf, 2015). The methods used for the development of CS NPs are either “bottom-up” or “top-down” techniques (Figure 1.8). Methods used to synthesise CS NPs using bottom-up techniques include ionic gelation (P. Calvo, 1997) and CS – lecithin NPs formation (M. P. Souza, et al., 2014).

Perhaps the most widely used method to synthesise CS NPs is ionic gelation (Calvo, Remuñan-López, Vila-Jato, & Alonso, 1997), a bottom-up process where an anionic crosslinker such as sodium tripolyphosphate (TPP) is added in a controlled manner to a solution of a known concentration of CS whereupon self-assembly of CS NPs occurs through the electrostatic interaction of protonated CS amine groups and the counter anions of TPP. While other anionic crosslinkers such as glutaraldehyde (a toxic substance) can also be used, TPP’s biocompatibility and biodegradability make TPP a crosslinker of choice for food applications. The factors that affect the particle size of self-assembled CS NPs are both intrinsic properties of CS (degree of deacetylation (DD), and molecular weight (MW)) and extrinsic properties (pH, concentration of CS and TPP and the CS:TPP mass ratio). Larger MW result in larger particle sizes due to the increased polymer chain length (O' Callaghan, et al., 2016) and larger CS NPs diameter are also obtained at pH's closer to 7 due to the reduced protonation of amine group while at pH's < 4.6 particle size increases due to the increased ionic strength of CH_3COO^- anions shielding interaction of protonated CS amine groups with anionic TPP, reducing the number of crosslinking sites and resulting in larger CS NPs (W. Fan, Yan, Xu, & Ni, 2012).

The initial CS concentration affect the nucleation mechanism of formation of CS NPs as higher CS concentration leads to the generation of many nuclei and these nuclei have higher growth rates due to repulsion between the CS chains resulting in the formation of bigger CS NPs (Rathi & Gaikar, 2017). It was reported that CS:TPP mass ratios of 1:1 have a larger size distribution due the presence of excess anions while lower CS:TPP mass ratios of 3:1 or 5:1 have an excess of cations yielding the most stable and monodisperse CS NPs (John Antoniou, et al., 2015; W. Fan, et al., 2012).

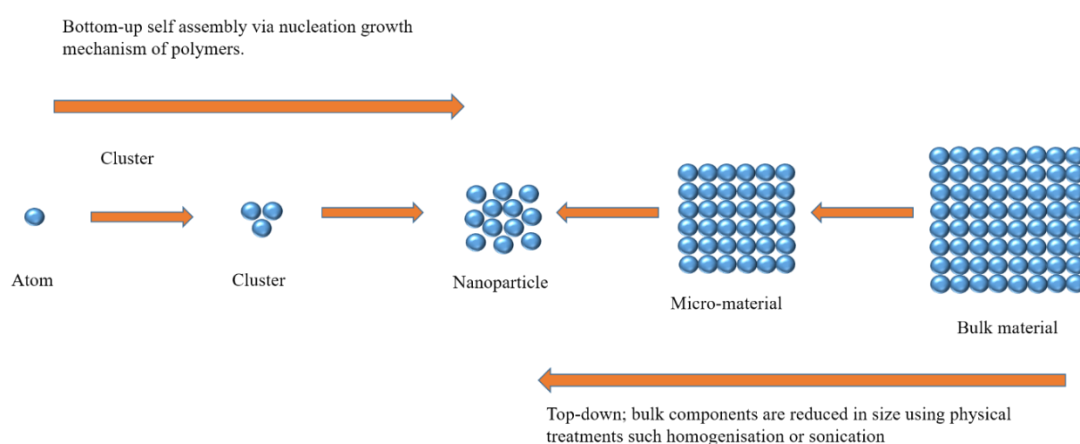


Figure 1.8 Schematic representation of bottom-up and top-down approaches to nanoparticle development.

For the synthesis of CS NPs using lecithin and CS, CS is dissolved into an aqueous acidic solution and lecithin dissolved in ethanol and then mixed using a 0.75 mm diameter needle whereupon CS – lecithin NPs self-assemble (Sonvico, et al., 2006). Souza, et al. (2014) developed quercetin loaded into CS-lecithin NPs which showed promising anti-oxidant activity in food systems.

The methods widely used for the synthesis of CS NPs using top-down techniques include spray drying (Gamboa, et al., 2015) and high energy sonication processes (Gokce, Cengiz, Yildiz, Calimli, & Aktas, 2014).

Spray drying of CS NPs is carried out by initially preparing a CS solution and then spray dried. The particle size of CS NPs can be controlled through manipulation of intrinsic and extrinsic properties of the process. Extrinsic properties included flow rate, nozzle size, temperature, drying gas flow rate and concentration of solutions while intrinsic properties such as MW also affected particle size development (Ngan, et al., 2014) of the CS NPs obtained using this technique have shown antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus*).

Another top-down high energy input technique used for develop CS NPs is sonication which can primarily be used breakdown CS particles that are either too large or have agglomerated over time. Sonication at 20 kHz frequency or above, breaks down CS into CS NPs *via* a mechanical process whereby bond breaking occurs at the $\beta(1,4)$ -linkage's due to the difference in bond energy between the between the glucosamine and N-acetyl glucosamine moieties (Gokce, et al., 2014). Physical breakdown of CS NPs occurs more efficiently when high molecular weights are used and at lower temperatures in conjunction with longer sonication times. Smaller CS NPs remain unaffected by this process, reducing potential damage to their structural integrity (John Antoniou, et al., 2015).

1.3.2.3 Intrinsic and Extrinsic Factors that affect Chitosan Antimicrobial Activity

CS antimicrobial activity varies considerably with the type of CS; particularly the concentration, molecular weight, degree of deacetylation (DD), the species of microorganisms and the conditions of the food matrix in which it is applied; especially pH, ionic strength and presence of solutes susceptible to react with CS through electrostatic interaction and/or covalent bonding which can screen or completely block the reactivity of the active amine group (Aider, 2010; Cruz-Romero, et al., 2013b; Kong, et al., 2010).

Studies have reported that the concentration of CS used will affect the antimicrobial activity of the CS (Cruz-Romero, et al., 2013b). CS concentrations higher than 200 ppm are required to have antimicrobial activity; however, concentrations below 20 ppm can stimulate microbial growth as CS's below concentrations of 20 ppm can be used as an energy source (N. Liu, et al., 2006).

Studies have also shown that the molecular weight of CS affects its' antimicrobial activity but a clear trend is yet to emerge in dependence. Largely, it has been reported that low molecular weight CS has better antimicrobial activity, as it has the ability to penetrate the cell wall of microbes and combine with their DNA, inhibiting the synthesis of mRNA and DNA transcription (Cruz-Romero, et al., 2013b; N. Liu, et al., 2006). Conversely, other authors have reported that increasing CS molecular weight of corresponded to an increase in antimicrobial activity against Gram-positive bacteria, which potentially form a film around the bacteria inhibiting nutrient uptake (S. H. Chang, et al., 2015). No, Park, Lee, Hwang, & Meyers, (2002) reported that antimicrobial activity of CS and CS oligomers varied depending on their molecular weights and the specific target bacterium in question.

The DD of CS affects the antimicrobial activity due to the greater number of amine groups that can be protonated. In general an increased DD was reported to have an increased antimicrobial activity; however, DD > 85 % did not significantly increase the antimicrobial activity of CS (J. Li, Wu, & Zhao, 2016). Similarly, Chung and Chen (Chung & Chen, 2008) observed increased antimicrobial activity against *S. aureus* and *E. coli* with increasing DD as an increased optical density due to nucleotide leakage was observed when the DD of CS was increased from 75% to 95%. Conversely, Qin, et al. (2006) reported that lower DD had better antimicrobial activity against *S. aureus* and *E. coli*.

Higher antimicrobial activity was observed when the pH of the surrounding environment approaches or goes below the pK_a of CS and this is explained by the degree of protonation on the amine group and its' relation to the antimicrobial activity. Generally CS antimicrobial activity is enhanced through the protonated amine in acidic solutions and protonation is reduced when the pH of the solution is increased (Kong, et al., 2010).

The Gram strain of the bacteria has been reported to affect CS antimicrobial efficacy. On Gram-positive bacteria, CS can form a linkage with lipoteichoic acid on the cell surface and disrupt membrane functions. It has been proposed that the antimicrobial activity against Gram-negative cells is due to that polycationic CS can compete with stabilising divalent metals (e.g. Mg^{2+} , Ca^{2+}) in the cell wall, leading to structural destabilisation (Cruz-Romero, et al., 2013b). Conflicting results have been reported on the antimicrobial activity against Gram-positive or Gram-negative bacteria, where some studies have indicated that CS had better efficacy against Gram-positive bacteria such *S. aureus* (Goy, Morais, & Assis, 2016) while other studies shown that CS had better antimicrobial activity against Gram-negative bacteria such as *Pseudomonas* sp. (Lopez-Caballero, Gomez-Guillen, Perez-Mateos, & Montero, 2005).

1.3.2.4 Mode of Antimicrobial Action of Chitosan

The broad spectrum of antibacterial activity associated with CS may be attributed to its ability to inhibit microbial growth by a number of different mechanisms which is dependent upon the microorganism of interest. The mode of antibacterial activity is a complex process that differs between Gram-positive and Gram-negative bacteria due to different cell surface characteristics (Kong, et al., 2010) and is affected by the afore mentioned intrinsic and extrinsic factors (section 1.3.2.3). Nevertheless, while exact

mode of antimicrobial action of CS is not fully understood (Rabea, et al., 2003), several modes of action have been proposed:

- i. Positively charged CS will interact with the negatively charged components of the bacterial cell wall, resulting in cytoplasm leakage from the microorganism (Dutta, Tripathi, Mehrotra, & Dutta, 2009).
- ii. Because of the strong chelating ability of chitosan, it is able to bind to metal ions such as Ni^{2+} , Fe^{2+} or Cu^{2+} present in the cell inhibiting the growth of the microorganism (Raafat & Sahl, 2009).
- iii. CS may also form a layer over the bacterial cell that will inhibit the absorption of nutrients, therefore, causing cell death (Kong, et al., 2010)).
- iv. Low molecular weight CS has been proposed to be able to bind to DNA and inhibit RNA replication due to its ability to penetrate towards the nuclei of microorganisms (Rabea, et al., 2003).
- v. The mode of action may also be affected by the bacterial Gram stain as high molecular weight CS can form a film on Gram-positive bacteria preventing uptake of nutrients while low molecular weight CS could interfere with cellular metabolism on Gram-negative bacteria due penetration of CS into the cell (Zheng & Zhu, 2003).

CS can also reduce lipid oxidation reactions can that cause food quality deterioration, including: off-odours, off-flavours, nutritional losses, colour or textural changes and these changes can significantly reduce the consumer acceptability of food products, increase the deterioration rate of food, decrease the shelf-life and lead to food losses (Lopez-de-Dicastillo, Alonso, Catala, Gavara, & Hernandez-Munoz, 2010; Tian, Decker, & Goddard, 2013).

Kim and Thomas (Kim & Thomas, 2007) studied antioxidant activity of CS of different molecular weights (30, 90 and 120 kDa) in salmon and found that the incorporation of 0.2%, 0.5% and 1% CS with various molecular weights into salmon reduced lipid oxidation. The 30 kDa CS which has high water solubility may be considered as a potential natural antioxidant for stabilizing lipid containing foods to prolong shelf life as well as being an excellent antimicrobial agent. The varying antioxidant effect of CS of different molecular weight in salmon was attributed to the molecular weight differences which determine the chelation of metal ions. It appeared that high concentrations of higher molecular weight CS were able to chelate metal ions or bind to the lipid and reduce its' potential for lipid oxidation. The antioxidant mechanism of action of CS is related to the metal-chelation ability of CS to form a complex with lipids and good scavenging ability on hydroxyl radicals and that the antioxidant properties are increased with the degree of N-deacetylation (Alishahi & Aider, 2012; Hamed, et al., 2016).

1.3.2.5 Packaging Applications of Chitosan in Food Products

Currently the most commonly used commercial application of CS is as a food ingredient and it is presently being used in Japan and South Korea in the manufacture of breads and noodles (You-Jin Jeon, 2002). CS can also be used in pork sausage formulations to reduce the microbial growth while also improving the texture via chelation of fat and water moieties (do Amaral, et al., 2015). CS has been also used in the manufacture of cod patties (Lopez-Caballero, et al., 2005).

CS has been used as polymer for the development of edible films or dip coating solutions that can directly coat onto food products to reduce microbial spoilage. Dip coating is defined as a technique when a food product is immersed into an antimicrobial solution for a period of time which then coats the food surface with a

thin film containing the antimicrobial. Silver carp (*H. molitrix*) coated with chitosan retained good microbiological, chemical (pH, TBA, TVB-N, K-value), and sensory quality characteristics and extended the shelf life of this products during frozen storage at -3°C (W. J. Fan, et al., 2009). Similar results were reported for eel fillets dip coated in aqueous solutions of the extracted and commercial chitosan. The coating did not affect significantly colour of eel fillets (except a^* values); however, significant positive effects on sensorial and microbial qualities were observed on chitosan coated eel fillets compared to control samples and an extended shelf life of eel fillets was obtained when products were coated with chitosan (Küçükgülmez, Yanar, Gerçek, Gülnaz, & Celik, 2013).

Edible coating have been applied onto various food products including strawberries (Han, Lederer, McDaniel, & Zhao, 2005), mangoes (Jongsri, Wangsomboondee, Rojsitthisak, & Seraypheap, 2016), papaya (Dotto, et al., 2015), muscle based products such as frozen ready-to-eat (RTE) shrimp (M. Guo, Jin, Scullen, & Sommers, 2013), oysters (Rong, Qi, Yin, & Zhu, 2010), salmon (B. W. Souza, et al., 2010), Herring and Atlantic cod (You-Jin Jeon, 2002), RTE chicken balls, chicken seekh kebabs and mutton seekh kebabs (Kanatt, Rao, Chawla, & Sharma, 2013) and pork (Darmadji & Izumimoto, 1994) among other products.

CS edible films can be manufactured through solvent casting and applied to food products; however, CS can also be combined with other biocompatible film forming polymers such as cellulose and/or NAMs. CS films containing a plasticiser (such as sorbital or glycerol) have been used to develop AAP films and it has been shown to have good antimicrobial activity against a wide range of spoilage and pathogenic microbes such as *L. innocua*, *L. monocytognees*, *S. typhimurium*, *S. aureus* and *E. coli*

among others (Pereda, Ponce, Marcovich, Ruseckaite, & Martucci, 2011; Rodriguez-Nunez, et al., 2012).

Chitosan-gelatin coatings and films have also been tested on products such as rainbow trout and Pacific white shrimp both stored under refrigerated conditions (Farajzadeh, et al., 2016). In Pacific white shrimp, the positive effects of a chitosan-gelatin coating led to a significant increase of sensory quality for an extended period of 6 days due to a significant decrease of total and psychotrophic bacteria and associated metabolites and a decrease in lipid oxidation (Farajzadeh, et al., 2016).

Typically CS films antimicrobial activity can be enhanced by combining with other NAMs. Albertos, et al. (2015) developed CS/Clove EO films and used to coat rainbow trout and the developed material significantly reduced the growth of spoilage microorganisms on trout thereby extending shelf life. Sharafati Chaleshtori, et al. (2015) manufactured CS based coatings incorporated with both eucalyptus and cumin EO's and applied on packaged fresh chicken. The results indicate that the CS-EO composite enhanced shelf life of chicken compared to control chicken without composite. Chinese mullet samples coated with CS, CS containing polyethyleneimine (CS/PEI) or thyme EO (CS/T) showed significantly lower microbial growth and delayed deterioration in quality parameters, such as pH, TVB-N, TBA, *K* value, sensory scores and texture. CS, CS/PEI and CS/T treatments extended fish shelf life by 4–5 days compared to control treatment; however, CS/PEI treatment exhibited superior preservation ability as this treatment maintained better the microbiological, physicochemical and sensory quality of the fish (F. Yang, et al., 2015).

CS has also been used as polymer in combination with commercial polymeric packaging materials to develop AAP. (Reesha, Panda, Bindu, & Varghese, 2015)

blended of 1, 3 and 5 % CS with LDPE and the LDPE- CS composite and extended the shelf life of tilapia to 15 days compared to 7 days for control LDPE without CS. Park, Marsh and Dawson (S. I. Park, Marsh, & Dawson, 2010) prepared 2 % CS – LDPE composites through a heat press (120 °C and 10 MPa for 90 s) and were applied to sliced red meats and observed that while there was no statistical significance difference in antimicrobial activity between the CS-LDPE and LDPE after 10 days storage; however, the redness of the meat was preserved through the chelation of CS with ferric iron (Fe^{3+}) in metmyoglobin, therefore, reducing its' ability to promote oxidation in red meat. Bie, et al. (2013) manufactured PLA/starch/CS in a 54/36/10 % ratio and has good antimicrobial activity against food borne pathogens *S. aureus* and *E. coli*. Bonilla, Fortunati, Vargas, Chiralt, & Kenny, (2013) manufactured CS - PLA composites using a twin screw extruder and the films were applied to extend its shelf life of pork mince. The samples packaged using CS-PLA films had significantly lower microbial spoilage compared to control PLA films without CS. Composite films of CS and polymeric packaging materials have been applied on food products such as tomatoes (Tripathi, Mehrotra, & Dutta, 2009), dried barberry (Valipour Motlagh, Hamed Mosavian, & Mortazavi, 2013), cheese (Peighambardoust, Beigmohammadi, & Peighambardoust, 2016) and beef steaks (Lago, et al., 2014) among others.

CS has also been used in the hurdle technology approach in combination with other mild preservation techniques. Several authors have reported the use of AAP in combination with vacuum packaging or MAP can be used to extend shelf life of food products. For instance, (Latou, Mexis, Badeka, Kontakos, & Kontominas, 2014) dip coated chicken breast fillets in a CS solution and then packed using MAP (70 % CO_2 / 30 % N_2). The shelf life of chicken breast fillets was significantly extended from 5 days to 9 days when used. Günlü, et al. (2013) wrapped sea bass fillets sea bass with

an edible CS film then vacuum packed in polyethylene film bags. The shelf life of samples vacuum packed with CS films had a shelf life of 25 days compared to control samples which have a maximum shelf life of 5 days.

As stated previously CS NPs favourable properties have seen it applied on foods such as: edible coatings (Pilon, et al., 2015), on cheese (O' Callaghan, et al.), coating shrimp (Y. B. Wang, et al., 2015) and as a carrier for other more volatile antimicrobials such as essential oils (Feyzioglu, et al., 2016) or as a feed additive for livestock (Y. Wang & Li, 2011).

1.3.4 Organic Acids

Organic acids occur naturally in many fruits or plants and are commonly used as a food preservative in the development of AAP. Commercially available organic acids include acetic, citric, lactic, succinic and tartic acids among others (Figure 1.9).

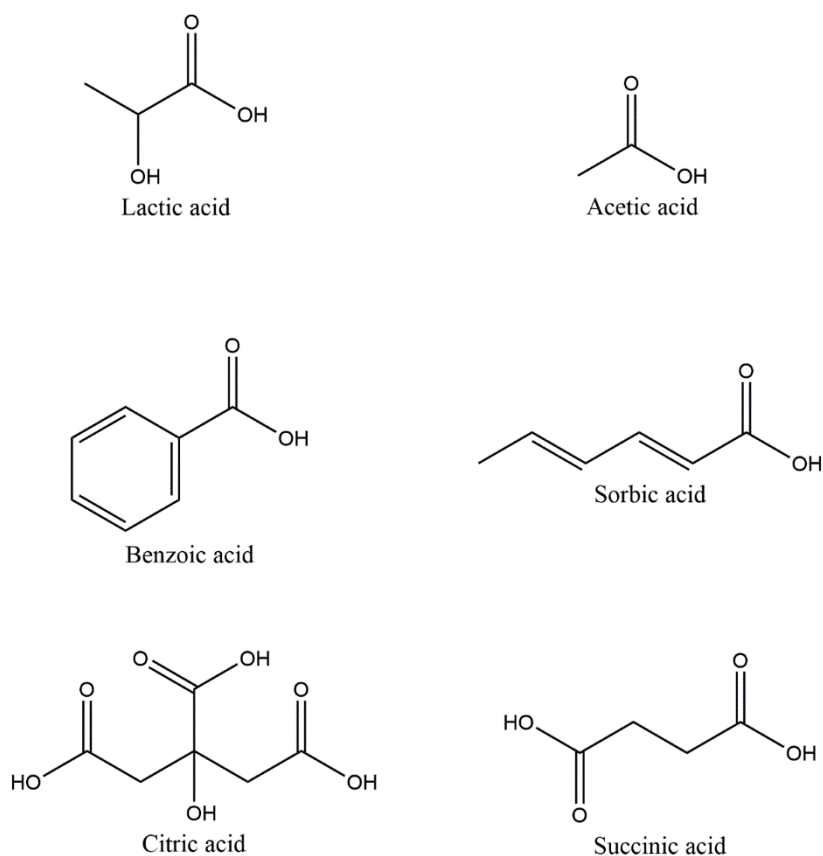


Figure 1.9 Commonly used organic acids in the food industry

However, sorbic and benzoic acids as well as their salts are the most frequently used. Combinations of organic acids commercially available include Articoat DLP 02 (sodium diacetate, lactic acid, acetic acid, citric acid, in pectin and water), Artemix 152/NL (sodium citrate, sodium ascorbate, and sodium metabisulphite) and Auranta FV (citric acid, malic acid, lactic acid and caprylic acid) and Novasol DC/44 (4 % sorbic acid and 4 % benzoic acid).

Benzoic acid and its' salt derivatives are currently used as antimicrobial preservatives in food products (such as preserves, fresh juices) and is recognised as “GRAS” by the FDA and as preservative E210 in the E.U. Concentrations of benzoic acid and sodium benzoate are allowed in food products are limited to 150 ppm and 5000 ppm respectively under European Union Directive 95/2/CE (Lucera, et al., 2014). Sorbic acid (trans, trans-2,4-hexadienoic acid) is a tasteless organic acid that is usually applied as a salt preservative in the food industry due to its poor solubility on products such as non-alcoholic drinks, dried fruits and pre-packaged vegetables. Sorbic acid is recognised as “GRAS” by the FDA and as a preservative in the E.U. (E202). The concentration of sorbic acid permitted in food products is limited to 300 ppm under European Union Directive 95/2/CE (Berger & Berger, 2013; Jipa, Stoica-Guzun, & Stroescu, 2012).

1.3.4.1 Factors that affect Antimicrobial Activity of Organic Acids

The pH of the food matrix will affect the antimicrobial activity of organic acids, where low pH solutions can enhance antimicrobial activity due to the ability of the acids to dissociate below their pKa value; however, dissociation of acids do not occur when pH of the food matrix is close to the pH of the internal cytoplasm (Mani-López, García, & López-Malo, 2012). As in other NAMs, Gram strain type affect the antimicrobial activity of organic acids on food products where Gram-negative type bacteria are less

susceptible than Gram-positive, potentially due the more complex cell membrane structure (Clarke, et al., 2016).

The molecular weight of the organic acid has also been shown to affect antimicrobial efficacy where smaller molecular weight organic acids predominantly are antimicrobial through the “weak acid” preservative theory; however, larger molecular weight organic acids interact with bacterial cell walls. It is more likely that these acids are active through lipophilic interactions with the cell membrane as they are too large to rapidly pass through pores in cell membrane (Mani-López, et al., 2012).

Particle size has also been demonstrated to affect the antimicrobial activity of the organic acids. Cruz-Romero, et al. (2013b) compared the antimicrobial activity of nano-solubilisates of benzoic acid and sorbic acid and their non-nano counterparts against food spoilage microorganisms (*B. cereus*, *S. aureus*, *E. coli* and *P. fluorescens*) and found that nano-solubilisates had greater antimicrobial activity compared to its’ bulk counterparts. This was attributed to particle size of the nano-solubilisates smaller which allowed a greater interaction with bacterial cell walls and that the amphiphilic properties of both benzoic and sorbic acid nano-solubilisates allowed interaction with both lipophilic and hydrophilic parts of the cellular organelles.

1.3.4.2 Antimicrobial Mode of Action of Organic Acids

The antimicrobial activity of organic acids are thought to exert their toxic effects through a variety of mechanisms (Halstead, et al., 2015). Weak acids can cross bacterial membranes more readily than strong acids, because of the equilibrium between their ionised and non-ionised forms, the latter of which can freely diffuse cross hydrophobic membranes causing to collapse the proton gradients that are necessary for ATP synthesis, as free anions will combine with periplasmic protons

pumped out by the electron transport chain, and carry them back across the membrane without passage through the F1Fo ATP synthase (Halstead, et al., 2015).

As the pH of the organic acids environment decreases towards the pK_a of the acid, its' antimicrobial effectiveness increases, as decreasing pH allows more protonation of the acid (and is in a pH dependant equilibrium). This reduces the polarity of the acid allowing its rapid diffusion through the cell membrane whereupon entering the alkaline internal pH of the cell, the internalised acid dissociates, increasing the concentration of protons and ions in the microbe, acidifying the cytoplasm, which in turn can cause acid-induced protein unfolding, membrane and DNA damage affecting cellular functions such as glycolysis, cell signalling and active transport (Halstead, et al., 2015; Mani-López, et al., 2012).

Organic acids that use the weak preservation theory include benzoic acid and acetic acid. However, not all organic acids follow this mechanism, for example sorbic acid does not release enough protons for this type of inhibition and therefore, it is plausible that its hydrophobicity can interact with the cell membrane affecting its permeability resulting in intracellular leakage (Mani-López, et al., 2012). Chelation of macronutrient metals such as Ca^{2+} by citric acid have also been shown to reduce the ability of the bacteria to thrive (Graham & Lund, 1986).

1.3.4.3 Food Packaging Applications of Organic Acids

Organic acids have been used to develop AAP systems such as dip coatings, edible films, polymeric packaging materials and these AAP materials have been used in combination with other preservative technologies as an additional hurdle. Lucera, et al. (2014) dip coated a 3 % potassium sorbate using sodium alginate as a carrier onto

mozzarella cheese and observed that this could extend shelf life by an extra 4 days compared to uncoated controls.

Battisti, et al. (2017) spray coated a biopolymeric solution of gelatin crosslinked with transglutaminase enzyme, containing glycerol and citric acid onto paper sheets and subsequently used to wrap minced beef hindquarters. The microbiological analysis of the beef packed using AAP and stored at 4 °C showed after 4 days the microbial load was ~ 40% less than control beef samples. Jipa, Stocia-Guzun and Stroescu (Jipa, et al., 2012) developed monolayer films, using powdered bacterial cellulose and PVA and were coated with another bacterial cellulose membrane to obtain multilayer films. These films showed antimicrobial activity against *E. coli* (K12-MG1655) suggesting promising applications as a food packaging material. Silveira, Soares, Geraldine, Andrade, Botrel, et al. (2007) solvent casted cellulose polymer films containing sorbic acid (0-6%) and then applied on pastry dough. After 40 days of storage, the pastry dough layered 3–6% of sorbic acid had significantly smaller microbial growth than, or equivalent to, the control pastry dough (potassium sorbate layered with LDPE films).

Synergistic effects of the organic acids have been demonstrated and currently a variety of commercial formulations for applications on a wide range of food products are available in the market. Clarke, et al. (2016) made beef-derived gelatine film incorporated with commercially available mix of organic acids Articoat DLP 02, Artemix Consa 152/NL, Auranta FV and sodium octanoate (Caprylic acid salt) and tested against a range of pure culture of *B. cereus*, *P. fluorescens*, *E. coli*, *S. aureus* and microflora isolated from commercial beef steaks. From all the antimicrobials tested, films containing sodium octanoate was the most effective antimicrobial against the tested microorganisms and these films can be used as antimicrobial coating for

conventional plastic-based food packaging. However, *in vitro* cultures were more susceptible to antimicrobials than food matrixes as the antimicrobials interact with the lipids and proteins present in food.

Organic acids have also been used in conjunction with other antimicrobials to enhance shelf life of food products (hurdle approach). Schirmer, et al. (2009) packed fresh salmon with CO₂ in brine with both citric acid and acetic acid and cinnamaldehyde and observed that no significant microbial growth after 14 days chilled storage (4°C compared to untreated salmon). Wan Norhana, Poole, Deeth, & Dykes, (2012) used a combination of nisin, EDTA, and organic acids salts (sodium benzoate, potassium sorbate and sodium diacetate) with vacuum packaging on shrimp and found that during chilled storage (4°C) for 7 days the microbial spoilage was lower than that of control samples (vacuum packed only). Blana, Polymeneas, Tassou, & Panagou, (2016) packed fermented olives coated with brine (9%, w/v, NaCl), acidified with 2% (w/v) citric acid and 1.5% (w/v) ascorbic acid into polyethylene (PE) pouches and stored at 4 or 20 °C for 357 days. It was observed that after this period the counts of *Lactobacillus pentosus* (B281) and *Lactobacillus plantarum* (B282) on olives were lower than control (only in PE pouch). Mastromatteo, Conte, Faccia, Del Nobile, & Zambrini, (2014) used a combination of MAP (75% CO₂ / 25% N₂) with alginate films loaded with potassium sorbate stored at 4 °C where shelf life of mozzarella cheese was extended to 160 days compared to unpackaged control shelf life of 52 days.

Hurdle technology including AAP containing organic acids on packaging materials, have been used extend the shelf life of a wide variety of products such as buffalo meat (Malik & Sharma, 2014), cooked turkey meat (Contini, et al., 2014), salami (Adela & Peter, 2014), sugar snaps (Van Haute, Uyttendaele, & Sampers, 2013), carrots (Pushkala, Parvathy, & Srividya, 2012), beef steak (La Storia, et al., 2012), French

beans (Gupta, et al., 2012), strawberries (Garcia, Pereira, Sarantopoulos, & Hubinger, 2012), baby spinach (Y. X. Huang & Chen, 2011) and pastry dough (Silveira, Soares, Geraldine, Andrade, & Goncalves, 2007) among others.

1.3.5 Bacteriocins

Bacteriocins are antimicrobial peptides derived from lactic acid bacteria (LAB) that have a bacteriostatic or bactericidal effect on other related and unrelated microorganisms (Balciunas, et al., 2013; Black, Kelly, & Fitzgerald, 2005; Rai, Pandit, Gaikwad, & Kovics, 2016) . Commercially available bacteriocins include pediocin, reuterin and lactacin; however, the most commonly bacteriocin used for food packaging applications is nisin due to its acceptance for commercial food applications and its' strong antimicrobial activity.

1.3.5.1 Nisin

Nisin, commercially known as nisaplin™, is a tasteless antimicrobial peptide obtained from several *Lactococcus lactis* strains which occur naturally in raw milk and fermented foods (Irkin & Esmer, 2015). Structurally nisin is made up of a 34 amino acid chains containing lanthionine and methylanthionine groups (Alishahi, 2014). Currently, nisin is the only bacteriocin with “GRAS” status from the FDA and in the E.U. is an approved additive (E234). Regulation of nisin in the E.U. limits it's use to clotted creams, mascarpone cheese, ripened foods and canned processed foods with a maximum concentration of 0.13 mg/kg (European Food Safety Authority, 2006; Rai, et al., 2016; Saini, Sillard, Belgacem, & Bras, 2016) . While nisins antimicrobial activity is limited to Gram-positive food-borne pathogens including *staphylococci*, *bacilli*, *clostridia* and *Listeria*, it has been used as an additional “hurdle” to food packaging. The solubility and stability of nisin is pH dependent, where solubility and stability of nisin increases in low pH environments.

1.3.5.2 Factors that affect antimicrobial activity of Nisin

Nisin antimicrobial activity against food pathogens is dependent on the physiochemical composition of the food, where interactions of nisin with proteins and enzymes present in the food matrix will affect its antimicrobial efficacy (Balciunas, et al., 2013). Nisins antimicrobial activity is also affected by the pH environment, with increased antimicrobial activity when decreasing the pH of foods.

While nisin is not inherently antimicrobial against Gram-negative bacteria, studies have indicated that nisin used in combination with chelators and other NAMs showed enhanced antimicrobial activity. Chelating agents (such as EDTA) permeate the outer membrane of Gram-negative bacteria by extracting Ca^{2+} and Mg^{2+} cations which stabilise lipopolysaccharide structure in the cell wall, subsequently allowing nisin molecules to enter into the bacterial cell (Wan Norhana, et al., 2012).

1.3.5.3 Mode of Antimicrobial Activity of Nisin

The mechanism of nisin's antimicrobial activity is not fully understood; however, it is believed to be through a dual mechanism where terminal structures of nisin peptides interact with the cell membrane of Gram-positive bacteria creating pores in the membrane. This results in the leakage of low molecular weight compounds such as ions and phosphates from the bacterial cell. In addition, nisin can inhibit cell wall formation in bacterial cells through interference with biosynthesis of the peptidoglycan layer and this is independent of the pore formation mechanism (Hassan, Kjos, Nes, Diep, & Lotfipour, 2012; Saini, et al., 2016).

1.3.5.4 Applications of Nisin in Food Packaging

Much research has gone into the development of AAP systems containing nisin for inhibition of food borne pathogens. Moreover recent research has focused on its

application into food packaging materials such as edible films and polymeric packaging materials and used as an additional hurdle in the hurdle approach food preservation. .

Imran, Klouj, Revol-Junelles, & Desobry, (2014) developed films of propyl methylcellulose (HPMC), CS, PLA and sodium caseinate (SC) containing nisin. They determined that nisin in HPMC and SC films had a higher inhibitory effect against *L. monocytogenes* (CIP 82110) and *S. aureus* (CIP 4.83) due to the higher release profile of nisin from these films suggesting that these types of materials are suitable for prolonging shelf life of food products. Correa, et al. (2017) developed biodegradable polyhydroxybutyrate/polycaprolactone (PHB/PCL) films with organo-clays (Cloisite 30 B and 10A) as a carrier for nisin and applied to ham slices inoculated with *Lactobacillus plantarum*. After 28 days storage the *Lactobacillus plantarum* counts on sliced ham were 50% lower compared to control films without nisin indicating these materials have good potential for extending the shelf life of ham. Saini, et al., (2016) grafted nisin to cellulose nanofibers and found that the developed materials significantly reduced *B. subtilis* and *S. aureus* compared to controls films.

More recently, AAP containing nisin have been applied to food. Marcos, Aymerich, Garriga, & Arnau, (2013) inoculated the surface of sliced fermented sausages with *L. monocytogenes* and packaged with PVA films containing nisin. The author observed that after 90 days of storage samples showed a pronounced reduction of *L. monocytogenes* counts compared to controls without nisin and these, therefore, increased the safety of sliced fermented sausages. Hanušová, et al. (2010) coextruded polyamide/polyethylene film coated with a polyvinylidene chloride lacquer containing both nisin and natamycin. AAP containing nisin or natamycin showed inhibitory effect against *B. cereus* inoculated on the surface of the packaged soft cheese. AAP systems

containing nisin as an antimicrobial have been applied to extend the shelf life of a variety of food products such as strawberries (Duran, et al., 2016), dry cured ham (Hereu, Bover-Cid, Garriga, & Aymerich, 2012), bread (Balaguer, Lopez-Carballo, Catala, Gavara, & Hernandez-Munoz, 2013), cheese (Resa, Gerschenson, & Jagus, 2014) and milk (Black, et al., 2005).

Studies have shown nisin is effective as part of hurdle technology. Guo, Jin, Wang, Scullen, & Sommers, (2014) coated edible antimicrobial coating solutions containing chitosan, lauric arginate ester (LAE) and nisin on PLA films and used these in combination with flash pressurisation to inactivate *L. innocua* inoculated RTE deli meat and found that *L. innocua* was significantly reduced. Nisin has been also used in combination with high pressure treatment (Black, et al., 2005), vacuum packaging (Neetoo, et al., 2008) and MAP (Economou, Pournis, Ntzimani, & Savvaidis, 2009) to preserve milk, salmon and chicken respectively.

1.3.5 Comparison of Silver Nanoparticles and Food-derived Nanoparticles

Compared to CS NPs, which is one of the most effective NAMs, silver nanoparticles (Ag NPs) have shown greater antimicrobial activity against pure culture and food derived microflora. When CS NPs and Ag NPs were tested against Gram-negative and Gram-positive bacteria it was found that; up to 4.7 times and 1.5 times more CS NPs were needed respectively to exhibit an antimicrobial effect comparable to Ag NPs. While both CS NPs and Ag NPs have a wide range of antimicrobial activity, Ag NPs were shown to have better antimicrobial activity against Gram-negative than Gram-positive bacteria (Cruz-Romero, et al., 2013a; Cruz-Romero, et al., 2013b; O'Callaghan & Kerry, 2014). In general microflora derived from food such as: raw chicken, raw beef, cooked ham or cheese were more resistant than pure cultures and the resistance was dependent on the food from which the microflora was isolated. This

was explained by the fact that microflora derived from food are more complex systems with heterogeneous populations of Gram-positive and Gram-negative bacteria which may have different resistances to antimicrobials compared to pure cultures of bacteria (Azlin-Hasim, et al., 2016). The difference in antimicrobial resistances between Gram-negative and Gram-positive can be explained by the properties of the bacterial cell membrane which are highly specific to the respective bacterial strain (Azlin-Hasim, et al., 2016).

When Ag NPs and CS NPs were applied on real food products, a significant increase in the shelf life of these products was observed. Azlin-Hasim, et al. (2015) manufactured LDPE films containing 0.5 and 1 w/w% Ag via extrusion and applied these onto fresh chicken breasts fillets in combination with MAP (40 % CO₂:60 % N₂) to extend the shelf life of chicken breast fillets. These composite films were able to significantly increase the shelf life of chicken breast fillets from a control (without Ag) of 6 days to 8 days. Similarly, AAP systems have applied Ag NPs to extend the shelf life of a wide variety of food products including beef (Fernández, Picouet, & Lloret, 2010), rice (L. Li, et al., 2017) and eggs (Viswanathan, Priyadharshini, Nirmala, Raman, & Raj, 2016) among others. Likewise, CS NPs in AAP systems were able to extended shelf-life of food products as outlined in section 1.3.2.5.

When compared to NAMs, the enhanced antimicrobial activity of Ag NPs could be due to the multi-target mode of action reducing the probability of microbes developing antimicrobial resistance. While exact mode of Ag antimicrobial action is unclear, several mechanisms have been proposed (de Azeredo, 2013) such as: the gradual release of metal ions has been reported to inhibit ATP production and DNA replication (Maillard, et al., 2013), damage to the cellular membrane through electrostatic interactions with thiol, phosphates, hydroxyls, indoles or amine in the cell impairing

regular functions (Dallas, Sharma, & Zboril, 2011) and generation of reactive oxygen species (ROS) (Emamifar & Mohammadizadeh, 2015).

One of the main advantages of using Ag NPs in packaging materials is their thermal stability, which allows for their incorporation into polymeric packaging materials (such as polyesters, polyamides, and polyolefins) through commercially available techniques such as extrusion. While NAMs have several favourable properties such as biodegradability, biocompatibility, good film forming ability, GRAS status and acceptance from consumers (Irkin, et al., 2015), many NAMs are thermolabile affecting antimicrobial activity when processed using high temperature techniques (such as extrusion) (Turek, et al., 2013), subsequently making their application in food packaging materials more challenging. Nevertheless, recent developments in NAM surface coatings technologies and low temperature processing techniques (as outlined in sections 1.4.4 – 1.4.8) have made the use NAMs more feasible for the development of AAP systems, without adversely affecting the antimicrobial activity of these NAMs (Fang, et al., 2017).

It was reported that one of the main constraints of the commercial development of Ag NPs based AAP systems are regulatory issues due to the migration of these material into food matrices (Hannon, Kerry, Cruz-Romero, Morris, & Cummins, 2015; Realini & Marcos, 2014) as currently the maximum acceptable limit for silver in food is 0.05 mg/kg (European Food Safety Authority, 2011). While application and acceptance of Ag based AAP systems in E.U. remains low, a number of antimicrobial Ag-based AAP systems such as Biomaster®, AgIon® and Irgaguard® are commercially available in the USA and Japan (Realini, et al., 2014).

Studies have reported that Ag can migrate from food contact materials into food matrices in above levels currently allowed; however, the degree of migration will depend on the technology employed to coat/manufacture the AAP systems and the concentration of antimicrobial used. Furthermore, extrinsic environmental factor such as temperature and time will also affect release of antimicrobial substances into the food product (J. C. Hannon, et al., 2016; Joseph Christopher Hannon, et al., 2016).

1.4. Development of Active Packaging systems

Numerous strategies have been developed for the delivery of NAMs into AAP systems; however, the physicochemical characteristic of NAM such as volatility and thermal sensitivity must be taken into account. Depending on the intended delivery method of the NAM to the food product, the development of AAP systems can be grouped into four different categories (Fang, et al., 2017; Paola Appendini, 2002): 1) incorporation of antimicrobial substances into a sachets/pads; 2) direct incorporation into polymeric packaging matrix; 3) coating the antimicrobial onto packaging materials surface and 4) using inherently antimicrobial film forming polymers (such as CS).

1.4.1 Natural Antimicrobials incorporated into Sachets and Pads

Carrier/emitter sachets and pads involves the adsorption of an antimicrobial compound into a carrier sachet or pad, contingent on the physicochemical properties of the NAM used and the antimicrobial activity of the sachets/pads can be either in direct contact with food products or through the release of volatile antimicrobials into the packaging headspace (Kerry, et al., 2006; Otoni, et al., 2016). Emitting sachets can be used impregnated a wide range of antimicrobials and one commercially available product is EthicapTM which contains ethanol in silicon dioxide powder and released into the packaging headspace (Otoni, et al., 2016). Absorbent pads are widely used in modified

atmosphere food packaging to preserve food products through the absorption of exudates secreted from food (e.g. drip loss in meat products) which will allow the rapid proliferation of spoilage microorganisms due the high water activity. NAMs have been incorporated into pads to reduce microbial spoilage (Otoni, et al., 2016). Typically these sachet pad materials are made from plastics, such as perforated LDPE; however, gels, starch, calcium alginate beds or silica are also used as carriers (Kerry, et al., 2006; Otoni, et al., 2016). Oral, et al. (2009) sprayed an oregano EO and Tween 80 solution onto perforated polyethylene, cellulose, and polyethylene pads. These pads were used to wrap chicken drumsticks and stored at 4 °C and the results indicated that microbial spoilage was 25 % less at day 7 when compared to untreated control samples. The application of these types of packaging systems with a variety of food products can be seen in section 1.3.1.5.

1.4.2 Natural Antimicrobials incorporated into Polymeric Films

Techniques such as extrusion and solvent casting have been used to develop AAP. Extrusion is a commercial packaging manufacturing technique of choice for film manufacture, and biodegradable or non-biodegradable polymeric material can be used. However, this technique is suitable only for thermally resistant NAMs such as metal ions and their nanoparticles (Nur Hanani, Roos, & Kerry, 2014). Solvent casting requires that both the antimicrobial and polymer are soluble in the same solvent which is then mixed, casted and allowed to dry until a film has formed. This technique is suitable for heat sensitive NAMs such as EO's which lose functionality during processing at high temperatures. A selection of antimicrobial packaging materials manufactured with antimicrobial substances embedded in a polymer matrix, their antimicrobial activity against a variety of microorganisms and their application to

Table 1.3 Examples of various AAP films manufactured using solvent casting or extrusion, their antimicrobial activity and their application to increase the shelf life of food products.

Polymer used	Antimicrobial substances	Antimicrobial test	Method of fabrication	Microorganism inhibited	Antimicrobial effect	Reference
PP	Carvacrol, thymol	Agar diffusion method	Compression moulding	<i>E. coli</i> & <i>S. aureus</i>	8% the maximum inhibition	(Ramos, Jimenez, Peltzer, & Garrigos, 2012)
LDPE	Oregano, thyme	Agar diffusion method	Single screw extrusion	<i>E. coli</i> , <i>S. typhimurium</i> & <i>L. monocytogenes</i>	4% the maximum inhibition	(Valderrama, Andrea, & de Rojas Gante, 2012)
Gelatine	Grape fruit seed, green tea	Agar diluting method - Pork loin steak enriched bacteria	Solvent casting	<i>E. coli</i> & <i>L. monocytogenes</i>	~1 log reduction	(Hong, Lim, & Song, 2009)
Cellulose	Cinnamaldehyde, eugenol	Agar diffusion method, MIC	Solvent casting	<i>B.cereus</i> , <i>E. faecalis</i> , <i>L.monocytogenes</i> <i>M. luteus</i> , <i>S. aureus</i> , <i>A. hydrophila</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. Enteritidis</i> , <i>C. albicans</i> , <i>S. cerevisiae</i> & <i>Z. rouxii</i>	<i>A. hydrophila</i> and <i>E. faecalis</i> were the most sensitive to EO	(Sanla-Ead, Jangchud, Chonhenchob, & Suppakul, 2012)

Chitosan	Thyme, clove, cinnamon EO	Agar diffusion method	Solvent casting	<i>L. monocytogenes</i> , <i>S. aureus</i> , <i>S. enteritidis</i> & <i>P. aeruginosa</i>	<i>P. aeruginosa</i> & <i>L. monocytogenes</i> were the most resistant & most susceptible, respectively to EO	(M. Hosseini, Razavi, & Mousavi, 2009)
Methyl cellulose	Olive leave extract	Agar diffusion method - kasar cheese enriched bacteria	Solvent casting	<i>E. coli</i> & <i>S. aureus</i>	~1.9-2.2 log reductions	(Ayana & Turhan, 2009)
Carboxymethyl cellulose (CMC)	Potassium sorbate	Agar diffusion method	Solvent casting	<i>A. flavus</i> & <i>A. parasiticus</i>	4% the maximum inhibition zone	(Sayanjali, Ghanbarzadeh, & Ghiassifar, 2011)
PVA	Nisin	Shelf-life - fermented sausage enriched Bacteria	Solvent casting	<i>L. monocytogenes</i> , <i>Enterobacteriaceae</i> , & <i>LAB</i>	Reduction depends on the bacteria	(Marcos, et al., 2013)
PLA/pectin	Nisin	Agar diffusion method/liquid culture test	Twin-screw extrusion	<i>Lactobacillus plantarum</i>	Synergistic effect on with pectin/PLA + nisin, ~9 log reductions	(L. S. Liu, et al., 2007)
LDPE	Sorbic acid	Shelf-life of pastry dough	-	Psychotropic, filamentous fungi & yeasts	1 log reduction	(Silveira, Soares, Geraldine, Andrade, & Goncalves, 2007)

Corn zein	Gallic acid	Agar diffusion method	Solvent casting	<i>C. jejuni</i>	10% the maximum inhibition	(Alkan, et al., 2011)
PVA/zein/al-ginate	Enterocins	Shelf-life - cooked ham	Solvent casting	<i>L. monocytogenes</i>	Significant reduction of bacteria during storage time using VP	(Marcos, Aymerich, Monfort, & Garriga, 2007)
Polystyrene	Acrylic & maleic acids	Disk diffusion method, MIC	Solvent casting	<i>Klebsiella pneumonia</i> , <i>E. coli</i> , <i>Sarcina lutea</i> & <i>C. albicans</i>	PS containing maleic acids has better antimicrobial activity	(Haroun, Ahmed, El-Halawany, & Taie, 2013)
LDPE	MMT/Cu ²⁺	Dynamic shake flask test	Twin-screw extrusion	<i>E. coli</i>	A maximum reduction value of bacteria was obtained at 4% Mt/Cu ²⁺	(Bruna, Penaloza, Guarda, Rodriguez, & Galotto, 2012)
EVOH	Ag ⁺	Microdilution method	Solvent casting	<i>L. monocytogenes</i> & <i>Salmonella</i> spp.	MIC range of 0.01-0.1 ppm	(Martínez-Abad, Lagaron, & Ocio, 2012)
EVOH	Ag ⁺	High protein and low protein food enriched Bacteria	Solvent casting	<i>L. monocytogenes</i> & <i>Salmonella</i> spp.	Low protein food shown higher inactivation of bacteria than high protein food	(Martínez-Abad, et al., 2012)

PVC/LLDPE	Ag NPs	Liquid diffusion test	Dry blending	<i>E. coli</i>	LLDPE (84% reduction) exhibited lower antimicrobial activity than PVC (100% reduction)	(Chinkamonthong, Kositchaiyong, & Sombatsompop, 2013)
Sodium caseinate	Chitosan	Agar diffusion method, Diffusion-type assay, Tube-assay method	Solvent casting	(cheese, carrot, salami)	Inhibited the growth of mesophilic bacteria, psychrotrophic, yeasts & moulds Both Gram positive & Gram negative bacteria were inhibited when the incorporation of chitosan was above 1.4%	(Moreira Mdel, et al., 2011)
LDPE	Chitosan	Diffusion-type assay	Solvent casting	<i>L. monocytogenes</i> , <i>E. coli</i> & <i>S. enteritidis</i>		(S. I. Park, et al., 2010)
LDPE	Chitosan	Agar diluting method – shelf-life of slice red meat	Solvent casting	TVC	-	(S. I. Park, et al., 2010)

extend shelf-life of food products is shown in Table 1.3. For non-volatile antimicrobials to be effective when applied these types of packaging systems direct contact with the food surface is required, and since the NAM will have to diffuse from the packaging to the food, they need a relatively high concentration compared to the direct attachment onto the packaging surface which would require less NAM.

1.4.3 Edible Coatings and Films

Edible films and coatings are made from biodegradable, biocompatible and edible materials such as lipids (beeswax, shellac), polysaccharides (chitosan, alginate, cellulose) and proteins (casein, whey protein, gelatine) either individually or in combination to augment barrier properties (Lin & Zhao, 2007). Edible coatings are food grade suspensions which may be delivered by spraying or dipping directly onto the surface of the food product whereupon drying a clear thin layer over the food surface is formed. Edible films are film forming solutions that are usually cast over an inert surface, allowed to dry and then placed in contact with a food surface (Sanchez-Ortega, et al., 2014). Film formation of edible coatings and films on food products is dependent on several factors including coating characteristics (composition, chemical structure, viscosity of the coating solutions, coating thickness, degree of crosslinking), coating processing conditions (temperature, pH, type of solvent), and type and concentration of additives (emulsifiers, plasticizers, or cross-linking agents) (Aloui, et al., 2016).

To enhance functionality of edible coatings and films, NAMs are added, as NAM can be gradually released over time from these films and coatings. AAP edible coatings and films have been applied to preserve foods such as: strawberries (Garcia, et al., 2012), mango (Jongsri, et al., 2016), papaya (Dotto, et al., 2015) and muscle foods such as chicken, beef, pork and salmon (Antoniewski, Barringer, Knipe, & Zerby,

2007). Commercially available edible films include New Gem™, which contains spices and bilayer protein films that are used to enhance ham glaze and Coffi™, which is made from collagen nettings used to wrap boneless meat products (Sanchez-Ortega, et al., 2014). Applications of edible coatings and films on food products were reported in sections 1.3.1.4, 1.3.2.5, 1.3.3.3 and 1.3.4.4

1.4.4 Surface Functionalisation of Polymer Films for Natural Antimicrobial Attachment

Attachment of NAMs directly onto the surface of commercially used polymers such as, PP or LDPE is limited owing to the polymer's inertness and hydrophobic characteristics (Barish & Goddard, 2011). In order to maximise the attachment, grafting or immobilisation of the antimicrobial substance onto the surface of such commonly used polymeric films surface modification of the surface of the film is required (Goddard & Hotchkiss, 2007). Surface modification can be carried out using chemical modifications such as chromic acid or bromoacetic acid treatments or through physical treatments such as plasma treatment, corona discharge and UV/O₃ irradiation (Goddard, et al., 2007).

Surfaces of fresh or processed products are the most likely areas for microbial contamination, therefore, the application of film coating or direct dipping of antimicrobial substance to polymers surface can increase the antimicrobial efficacy due to direct contact of the NAM with the food product; thereby allowing direct contact or direct dispersion (or evaporation of volatile components) of the antimicrobial substance to the surface of food products at a high enough concentration to provide adequate antimicrobial activity. Previous studies have reported that incorporating antimicrobial substances onto polymer surfaces provided greater antimicrobial activity against a wide range of microorganisms compared to

antimicrobial substances embedded within the polymer matrix. Elsabee, Abdou, Nagy, & Eweis, (2008) irradiated PP films using corona discharge (1.1 kW, 380 V) and were immersed for 15 mins in 1% chitosan solution followed by an immersion for 15 mins in a 1% pectin solution, and repeated 15 times; then applied on tomatoes, which showed no visible rotting for 15 days. Shin, Liu, Chikthimmah, & Lee, (2016) grafted an acrylic acid monomer onto LDPE using photo-initiated graft polymerization using UV irradiation (250 – 320 nm) whereupon natamycin was applied to the treated LDPE surface. When these materials were tested on cantaloupe; the qualitative observation indicated effective mould inhibition.

Another advantage of using a coating or a dipping technique onto modified surfaces is that these methods do not affect heat-sensitive antimicrobial substances while the thermal or mechanical properties of the polymeric films are not significantly affected compared to untreated control films (Chen, et al., 2012). A selection of antimicrobial packaging materials manufactured using coating or dipping methods, their antimicrobial activities against various microorganisms and their application to extend the shelf-life of food products is shown in Table 1.4.

1.4.5 Layer-by-Layer attachment of Natural Antimicrobials

Layer-by-layer (LbL) involves the alternate deposition of species with complimentary chemical interaction (positively and negatively charged) to fabricate composite films and can be driven by multiple weak interactions, including van der Waals interactions, electrostatic interactions, hydrogen bonding, weak coordination bonding, etc. (Figure 1.10) (Dvoracek, Sukhonosova, Benedik, & Grunlan, 2009; Y. Li, Wang, & Sun, 2012). This technique is simple, easy to use, low cost, and adaptable to almost any kind of substrate and it is a flexible strategy of deposition to create antimicrobial film surfaces, making this technique a versatile nanofabrication method.

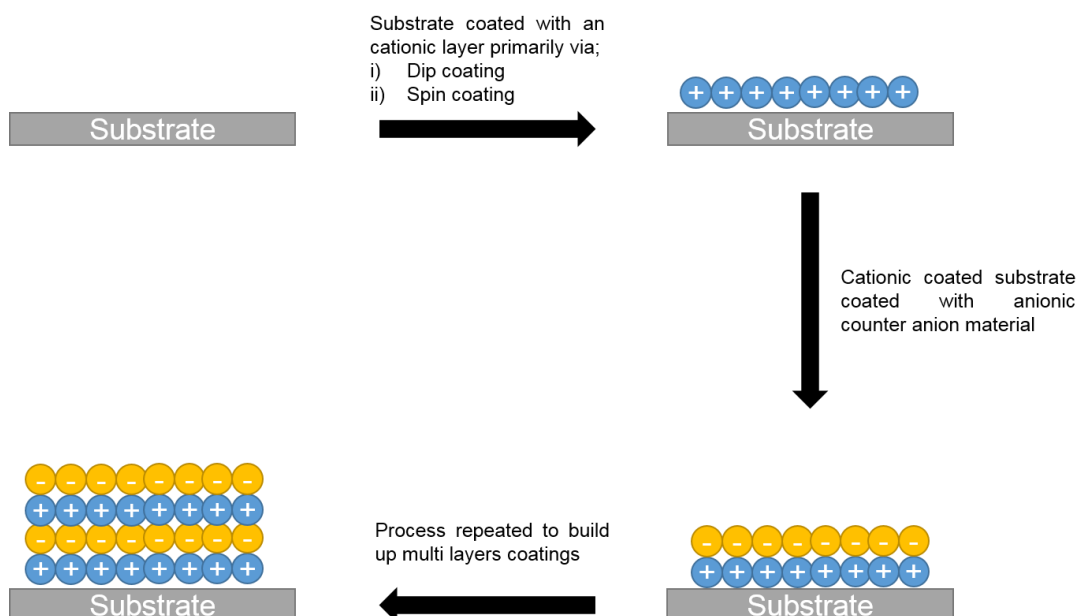


Figure 1.10 Schematic of LbL deposition on a substrate

By using the LbL strategy, the physicochemical characteristics of film surface such as thickness, chemistry, stability, gas permeability, mechanical properties and biofunctionality can be accurately controlled through adjustment of the assembly parameters such as temperature, ionic strength, concentration and the pH of the dipping solutions.

Huang, et al. (2012) developed electrospun cellulose acetate (CA) fibrous mats that were modified with multilayers of the positively charged lysozyme–chitosan–organic rectorite composites and the negatively charged sodium alginate via LbL self-assembly technique. When applied to pork samples, extended shelf life of an additional 3 days was obtained. Similarly, Poverenov, et al. (2014) coated a bilayer of CS and alginate on fresh-cut melon which after 11 days showed a reduction in microbial growth of two thirds compared to untreated controls.

1.4.6 Electro-hydrodynamic Coatings of Natural Antimicrobials

Electro-hydrodynamic techniques such as electrospraying and electrospinning have been utilised to develop novel AAP systems for food packaging applications. Electro-hydrodynamic techniques have an external high voltage electric field applied to a polymer solution in a capillary tube where a Taylor cone is formed at the nozzle and depending on the properties of the polymer solution either droplets (electrospraying) or a jet (electrospinning) is formed (Anu Bhushani & Anandharamakrishnan, 2014).

Electrospraying occurs when the physicochemical properties of the polymer solution have low viscosity, polymer chain entanglement and molecular weight resulting in the formation of monodisperse droplets. Electrosprayed droplets have been shown to have a higher surface area, controllable droplet size and release and can form thin layered coatings (Bock, Dargaville, & Woodruff, 2012). NAMs can be incorporated into electrosprayed droplets through the exogenous addition to the polymer solution before electrospraying (Echegoyen, Fabra, Castro-Mayorga, Cherpinski, & Lagaron, 2017). Moreover, electrosprayed droplets have been shown to encapsulate bioactive compounds that are thermolabile (such as EO's) without adversely affecting their functionality (Feng, et al., 2017). Morphological features and encapsulation ability of electrosprayed droplets can be controlled by changing operating parameters such as the flow rate and applied voltage and polymer parameters such as the solvent and concentration (Bock, et al., 2012).

Electrospinning is seen when the physicochemical properties of the polymer solution have higher viscosity, polymer chain entanglement and molecular weight resulting in the formation of jet which forms randomly aligned micro or nanofibre mats on the target surface (Anu Bhushani, et al., 2014). Fibres produced from electrospinning have favourable physicochemical properties such as: large surface area, enhanced

mechanical strength and high porosity. These features allow for value-added food packaging applications such as improved barrier properties of packaging materials and in the incorporation and delivery of NAMs where their release into the food matrix can be controlled (Lavielle, et al., 2013; Rezaei, Nasirpour, & Fathi, 2015). The properties of fibres produced by electrospinning are affected by the process parameters such as voltage and polymer flow, and the polymer solution properties such as: the degree of viscosity, molecular weight, polymer concentration, polymer entanglement and polymer conductivity (Anu Bhushani, et al., 2014; Rezaei, et al., 2015).

Rieger & Schiffman, (2014) electrospun chitosan/poly(ethylene oxide) nanofibre mats and loaded with 0 %, 5 % and 0.5 % cinnamaldehyde. These mats were tested against *P. aeruginosa*, where it was demonstrated that all nanofiber mats showed antimicrobial activity; however, nanofiber mats loaded with 5 % cinnamaldehyde exhibited the highest antimicrobial effect. Additionally electrospun NAMs have been applied to food products by authors such as Erbay, Dağtekin, Türe, Yeşilsu, & Torres-Giner, (2017) who electrospun poly(ϵ -caprolactone) (PCL) nanofibers containing extract of *Urtica dioica* L (common nettle) and incorporated this into whey protein isolate (WPI) films. These were applied to rainbow trout fillets, extending the shelf life and quality of trout fillets up to 15 days. Wen, et al., (2016b) electrospun PVA/cinnamon EO/ β -cyclodextrin antimicrobial nanofibrous films and applied it to fresh strawberries and found that the quality of the strawberries was maintained and shelf life extended by 2 days. In a different study the same author incorporated cinnamon EO/ β -cyclodextrin into PLA nanofibers via electrospinning and applied to fresh pork, doubling the shelf life of pork to 8 days (Wen, et al., 2016a).

1.4.7 Sol-Gel

Hybrid inorganic-organic materials are a promising system for a variety of applications due to their properties based on the combination of different building blocks. The ideal procedure for the generation of hybrid inorganic-organic materials is the sol-gel process which involves a molecular precursor as a starting material, followed by the formation of oxide frameworks by hydrolysis and condensation reaction (Kickelbick, 2003). The sol-gel process is particularly attractive because it presents advantages such as high purity of reactants and final products, mild processing conditions and it is possible to control connectivity and morphology by suitable choice of reactants, catalyst and reaction conditions. Additionally, it is transparent due to the nano-sized organic and inorganic domains, easy to apply on any kind of substrates and easy inclusion of suitable organic or inorganic of active substances for the preparation of functional hybrid materials (Marini, De Niederhausern, et al., 2007).

The disadvantage of the sol-gel process is that long reaction times are required to achieve a high cross-linking degree as sol-gel reactions are carried out under mild conditions. However, Geppi, et al. (2007) has proposed that the long reaction times of sol-gel could be reduced from 7 h to 1 min using microwave heating. The use of sol-gel has the potential to modify the surface of polymer films without using any pre-treatment and the coated materials have good adhesion onto the polymer film surface.

Table 1.4 Examples of various antimicrobial films manufactured using coating or dipping methods, their antimicrobial activity and their applications to increase the shelf-life of food products.

Material	Antimicrobial substances	Casting technique	Antimicrobial test	Method of surface modification	Microorganisms inhibited	Antimicrobial effects	Reference
PET/PP	Sodium benzoate, potassium sorbate, calcium propionate & ϵ -poly-lysine	Dipping	Liquid culture test	Atmosphere pressure plasma treatment	<i>S. aureus</i> , <i>B. subtilis</i> & <i>E. coli</i>	Good antimicrobial activity against <i>E. coli</i> & <i>B. subtilis</i> but poor against <i>S. aureus</i>	(Lei, et al., 2014)
PP	Propolis/chitosan	Coating	Quantify the survival of bacteria held in intimate contact	Corona treatment	<i>B. cereus</i> , <i>L. monocytogenes</i> , <i>S. aureus</i> , <i>Cronobacter sakazakii</i> , <i>S. typhimurium</i> & <i>E. coli</i>	3.87-5.58 log reductions	(Torlak & Sert, 2013)
LDPE	Sorbic acid/lacquer	Coating	Cheese & pork enriched bacteria	-	<i>E. coli</i>	~1 log reduction	(Hauser & Wunderlich, 2011)

LDPE	Sorbic acid/lacquer	Coating	Liquid culture test	-	<i>E. coli</i> , <i>L. monocytogenes</i> & <i>Saccharomyces cerevisiae</i>	5.4-6.5 log reductions	(Hauser, et al., 2011)
PP/EVOH	Lys	Bioconjugation	Liquid culture test/MIC	UV irradiation	<i>Micrococcus lysodeikticus</i> & <i>L. monocytogenes</i>	0.95-1.34 log reductions	(Muriel-Galet, Talbert, Hernandez-Munoz, Gavara, & Goddard, 2013)
PET	Lys	Sol-gel deposition - dip coating	Agar diffusion method	Cold plasma	<i>Micrococcus lysodeikticus</i>	Higher DIZ with the presence of Lys	(Corradini, et al., 2013)
Corn starch	Peptide dermaseptin	Coating	Shelf-life - Cucumber	-	Moulds & Aerobic bacteria	~1.3 log reductions	(Miltz, Rydlo, Mor, & Polyakov, 2006)
LDPE	Nisin	Coating	Agar diffusion method & milk enriched bacteria	-	<i>Micrococcus luteus</i>	0.9-1.3 log reductions	(Mauriello, De Luca, La Storia, Villani, & Ercolini, 2005)

PP	Nisin/protein	Coating	-	Corona discharge	-	-	(Lee, Son, & Hong, 2008)
HDPE	Nisin/organic acid/EDTA	Coating	Shelf-life - beef steak	-	TVC, <i>Enterobacteriaceae</i> , LAB, <i>Pseudomonas</i> spp. & <i>B. thermosphacta</i>	~1 log reduction of TVC & > 3 log reductions with MAP	(La Storia, et al., 2012)
Surlyn®	Nisin-chitosan	Coating	Smoked salmon enriched bacteria	-	<i>L. monocytogenes</i>	0.9-2.1 log reductions	(Ye, Neetoo, & Chen, 2008)
Zein/fibre	Chitosan	Electrospinning	Liquid culture test	-	<i>S. aureus</i>	> 4 log reductions	(Torres-Giner, Ocio, & Lagaron, 2009)
LDPE	Enterocin	Sol-gel	Agar diffusion assay/Liquid culture test /food contact assay frankfurters & cheese	-	<i>L. monocytogenes</i>	1.5 log reductions after 72 h for liquid culture test, & decreased bacterial growth during storage of frankfurters & cheese	(Iseppi, et al., 2008)

Corradini, et al. (2013) dip coated cold plasma treated PET films vertically at a constant speed of 7.0 cm/min into a sol-gel containing lysozyme. These films showed inhibition of *Micrococcus lysodeikticus* on agar plates at concentrations of 1.25 mg/ml indicating significant potential for the use of these packaging materials as a food preservative. Lantano, et al. (2014) developed PLA films activated using a sol-gel process, employing tetraethoxysilane as a precursor of the inorganic phase and polyvinyl alcohol as the organic component, and incorporating natamycin as the active agent. Activated PLA films were applied on cheese where after 30 days storage at 4 °C, no mould growth was observed on the cheese surface, compared to extensive mould growth on unactivated PLA films.

1.4.8 Aerosolisation

Aerosolisation is defined as the dispersion of a liquid phase into air, in the form of fine mist, usually for sanitary purposes, especially for respiratory medical treatments and room disinfection (Andersen, et al., 2006). This type of delivery offers a number of advantages including relative ease of application and higher surface coverage and penetration due to the small droplet compared to conventional spray coating size; however, roadblocks include the scaling up the aerosolisation unit and the limited number of antimicrobials that can be used (Y. B. Jiang, et al., 2017). Nonetheless, nebulizers are a readily available aerosolisation system which are widely used as a delivery apparatus for drugs into the lungs through the generation of a fine “mist” which typically have an average droplet diameter of $\geq 5 \mu\text{m}$ and is a smaller droplet than conventional spray coated generated mist (S. H. Park, et al., 2012).

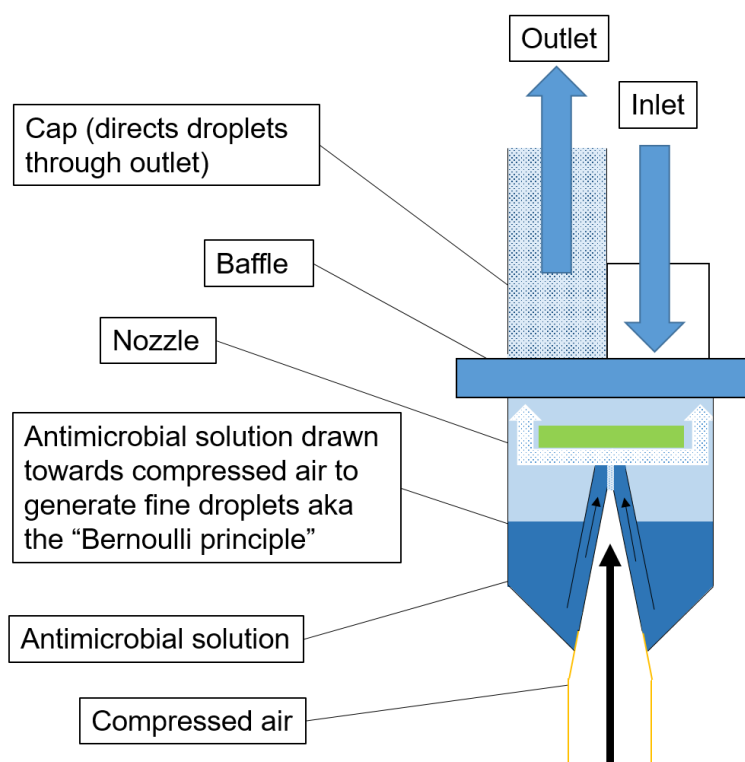


Figure 1.11 Schematic of a jet nebulizer cup

The most commonly used nebulizer are atomisers (also known as jet nebulizers) and the working principle of an atomiser is based on Bernoulli principle by which compressed gas (air or oxygen) is passed through a narrow orifice, creating a low-pressure area at the adjacent liquid feed tube. This evacuates the solution, with the antimicrobial being drawn up from the reservoir solution and aerosolises the antimicrobial materials into fine droplets (Figure 1.11) (Mukherjee, et al., 2017).

Currently, most studies involving aerosolisation treatment systems have focused on organic acid sanitisers and disinfectants such as peroxyacetic acid and sodium hypochlorite due to their water solubility and broad antimicrobial activity and have been applied on to various fruits such as cherry tomatoes (Y. B. Jiang, et al., 2017) and strawberries (Vardar, Ilhan, & Karabulut, 2012). However, nanotechnology may allow for the increase in scope of materials that could be applied via aerosolisation due to their small size features which can be easily dispersed as a particle, colloid or

micelle in an aqueous solution. Nanotechnology is a broad interdisciplinary area of research, development and industrial activity that involves the manufacture, processing and application of materials that have one or more dimensions of the order of 100 nanometres (nm) or less (Chaudhry, et al., 2008).

1.5. Conclusions

Considerable progress has been made in developing NAMs as active agents for extension of the shelf-life of foods. Herein this chapter has summarised the field to date. It is clear that there are a number of potential strategies and materials for achieving significant effects. However, one of the difficulties in this field is making direct comparisons of work from different laboratories since there is no uniform method of testing and no agreed standards on which to base quantitative comparisons. Furthermore, the possibility of a proper comparison is more complex because the spectrum of NAMs antimicrobial activity against pathogens varies depending in the climate in which they were grown and their geographical source (Calo, et al., 2015). Thus, it is hard to identify the most effective NAMs or combination thereof.

The commercial uptake of AAP as an effective technology for reducing spoilage is somewhat limited. This is partly because of lack of quantitative data, a variety of processing methods and some of the challenges these bring, as well as both product and capital equipment costs. However, there are examples here where the benefits in terms of reducing spoilage and maintaining freshness can be cost-effective. There are clear choices to be made, can these NAMs be directly applied to the food (Dotto, et al., 2015), applied into storage pads (Otoni, et al., 2016) or directly applied to packaging materials using either inclusion (Javidi, et al., 2016) or coatings (Goddard, et al., 2007). For effective inclusion of materials into polymers, the NAM and the polymer must share similar solubility parameters (Paola Appendini, 2002). If it is not

included in the polymer processing process (e.g. extrusion) than solvent swelling may be used but this may have a deleterious effect on the optical, barrier and mechanical properties of the polymer (Aloui, et al., 2016).

Despite technical roadblocks, more widespread use of NAMs is expected. As, for example, food security becomes ever more urgent, legislation increases and customer demand increases for fresh food, the need to find solutions to these roadblocks will become more pressing. However, for the reasons given above, there will be no single solution but instead a myriad of methods targeting product type, location and export/domestic requirements. Niche applications will provide entry points to markets and there is the potential for innovation and the development and start-up of small and medium enterprises where the market can bear the cost of price differentials between the NAM technology and the established materials. There will be a constant need to improve both the materials (by combination with other NAMs, through size optimization, material properties (such as molecular weight, purity etc.)) as well as processing (coating, extrusion techniques etc.). Whilst this is becoming a well-established area of research, it is only to be expected that the research effort will be magnified in both academic and industry laboratories.

1.6. Thesis Aims and Rationale

The aim of this thesis was to develop natural antimicrobial nanomaterials from food-derived sources and to apply these antimicrobial materials onto food products to enhance shelf-life. Currently, one of the main methods for food preservation is through the use of chemical preservatives but the use of these preservative have come under increased scrutiny due to their potential to have long term adverse effects on health (e.g. nitrate and nitrites). Similarly, metal ion nanoparticles as antimicrobial agents in food systems have been widely reported on; however, an increased number of studies

have shown higher health risks associated with the long term use of metal ion nanoparticles. Consequently, consumer demand has begun to move towards fresh like, minimally processed food products; however, removing chemical preservatives will subsequently reduce the shelf-life of food products. Therefore, to overcome this challenge, materials derived from natural sources such as: plants or crustacean shells, could be used. These natural materials have many favourable properties including: broad antimicrobial activity, biocompatibility, biodegradability, and will typically have “Generally Recognised as Safe” (GRAS) status.

In order to determine the most suitable materials, a state of the art literature review (Chapter 1) was carried out and upon completion; chitosan, oregano essential oil and commercially-available organic acids were identified as the most suitable materials to develop into nanomaterials. In chapter 2 we discuss the development of chitosan nanoparticles using bottom-up and top-down methods. Chapter 4 is concerned with the development of nanoemulsions of oregano using different surfactants, surfactant-to-oil ratio's and the effect of various physical forces on the size properties of the nanoemulsions. Finally in chapter 6, we use commercially available nano-solubilisates of organic acids and assess their antimicrobial effect against both planktonic and biofilm cultures.

While these chapters highlight that NAM's have good antimicrobial activity against a range of Gram-negative and Gram-positive microorganisms, the next challenge was to incorporate these antimicrobial materials into a food packaging systems. Unlike metal ion nanoparticles, NAMs are more thermolabile and can be sensitive to other environmental factors such as light. Therefore, an “aerosolisation” strategy was adopted to incorporate the developed materials into food products. Hake fillets were chosen as the food product due to their short shelf life, highly perishable nature and

the wide variety of microorganisms that exist on fish products. To the best of our knowledge, none have reported on the application of NAM nanomaterials on fish products elsewhere. In fact, this method several advantages over conventional coating methods as firstly: it uses small volumes of antimicrobial solution, has good coverage on the food product surface, and is directly applied to the food surface without affect the proximal composition properties of the food product. The application of CS materials using aerosolisation, as part of a hurdle strategy, was covered in chapter 3 while the application of oregano nanoemulsions was covered in chapter 4. Finally in chapter 5, an alternative method to incorporate oregano into food systems was investigated. In this method, the oregano essential oil was directly loaded into a siliceous reservoir that has been attached to a surface.

Overall, this thesis aims to develop various natural antimicrobial nanomaterials for food packaging applications and in particular:

- Develop nanomaterials from different NAM sources and to assess their physicochemical properties
- Assess their antimicrobial activity against common Gram-negative and Gram-positive food spoilage microorganisms.
- Develop an aerosolisation method for the NAM treatment of food products as part of a hurdle strategy
- To investigate the shelf-life enhancement using aerosolisation as an antimicrobial active packaging technology
- To develop a siliceous reservoir attached to a surface, for oregano oil loadings
- To investigate the effect of commercially-available organic acid nano-solubilisates against planktonic and biofilm cultures

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Chapter 2: Synthesis of Monodisperse Chitosan Nanoparticles

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This Chapter was published as:

“Sullivan, D. J., Cruz-Romero, M., Collins, T., Cummins, E., Kerry, J. P., & Morris, M. A. (2018). Synthesis of monodisperse chitosan nanoparticles. *Food Hydrocolloids*, 83, 355-364..”

2.1 Abstract

The objectives of this study were to evaluate the effects of the initial concentrations of chitosan (CS) and sodium tripolyphosphate (TPP), the CS:TPP mass ratio, the CS molecular weight (MW) and pH on the synthesis of CS nanoparticles (CS NPs). The particle size of the synthesised CS NPs was significantly affected ($P<0.05$) by these parameters. Self-assembled monodisperse CS NPs with a particle size of 90 nm and zeta potential of 30.15 m were successfully synthesised using solutions of 0.1% low MW CS at pH 4.6 and 3:1 (CS:TPP) mass ratio. When higher concentrations of CS were used, application of external forces (tip sonication or Ultra-Turrax™) was necessary to induce monodispersity and significantly ($P<0.05$) reduce the particle size; however, the particle sizes were >300 nm. While both native CS and CS NPs showed antimicrobial activity, no significant antimicrobial enhancement was observed for the NP form. The findings of this study have shown that monodisperse CS NPs can be obtained using a combination of bottom-up and top-down techniques and the unique physicochemical properties of these nanomaterials have the potential for applications in developing of antimicrobial active packaging materials.

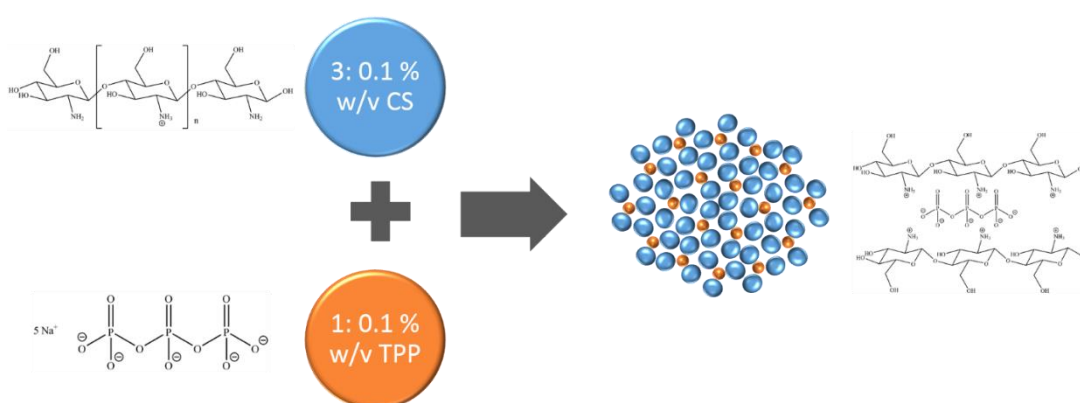


Figure 2.1 Graphical representation of chitosan nanoparticles formation

2.2 Introduction

Numerous natural antimicrobials (NAMs) have been identified and applied in the food industry (Sullivan, et al., 2018). However, of these NAMs, chitosan's (CS) numerous

favourable properties such as GRAS status, biodegradability, biocompatibility and antimicrobial properties against a wide range of spoilage and pathogenic microorganisms along with its relative abundance and low cost have seen a significant increase in its applications in the food industry (Kong, Chen, Xing, & Park, 2010; Madureira, Pereira, Castro, & Pintado, 2015; Paomephan, et al., 2018; Ryan, et al., 2016). Structurally CS is made up of a random assortment of $\beta(1-4)$ N-acetyl-D-glucosamine linkages where the ratio of the D-glucosamine to N-acetyl-D-glucosamine affects the physicochemical properties of bulk chitosan (Souza, Campiña, Sousa, Silva, & Gonçalves, 2013). While the antimicrobial mechanism of CS is not fully understood, several theories have been suggested that CS can either: electrostatically interact with negatively charged components of bacterial cell walls (Pilon, et al., 2015) or through an ability to bind to DNA and inhibit RNA replication (Cruz-Romero, Murphy, Morris, Cummins, & Kerry, 2013). Moreover, studies have shown that the antimicrobial activity of CS is affected by intrinsic and extrinsic factors such as CS concentration, molecular weight (MW), degree of deacetylation (DD), pH and particle size (Antoniou, et al., 2015; Kheiri, Moosawi Jorf, Malihipour, Saremi, & Nikkhah, 2016; Ngan, et al., 2014).

Current research has been focused on the development of CS NPs for a wide range of applications in the food industry. CS NPs can be synthesised using either “bottom-up” or “top-down” techniques. Bottom-up is the self-assembly of atoms and molecules into larger nanoscale compounds (Sanguansri & Augustin, 2006), whereas a top-down approach is the breakdown of larger compounds into smaller nanoscale compounds through an external physical and/or chemical force (Ma, Liu, Si, & Liu, 2010). Studies have found that CS NPs can be developed through several different “bottom-up” approaches such as spray-freeze-drying which produce CS NPs (Gamboa, et al.,

2015), through reverse micelle medium methods (Kafshgari, Khorram, Mansouri, Samimi, & Osfouri, 2012) or through the polymerisation of CS with methacrylic acid (PMAA) to make CS-PMAA NPs (de Moura, Aouada, & Mattoso, 2008). Top-down techniques have also been employed such as the wet milling process (Zhang, Zhang, & Xia, 2012) and high intensity ultrasound (Souza, et al., 2013).

Currently the most widely used synthesis of CS NPs is through ionic gelation (Calvo, Remuñan-López, Vila-Jato, & Alonso, 1997), a bottom-up process whereby an anionic crosslinker such as sodium tripolyphosphate (TPP) is added to a solution of CS whereupon CS and TPP self-assemble into CS NPs. While many anionic crosslinkers such as glutaraldehyde (a toxic substance) can be used, TPPs favourable properties such as its biocompatibility and biodegradability make TPP a more suitable crosslinker for food applications. A facile top-down approach to particle size reduction is through the application of high intensity sonication techniques which reduce the overall particle size either through physical breakdown (cavitation mechanism) (Gokce, Cengiz, Yildiz, Calimli, & Aktas, 2014) or through free radical initiated polymer degradation (random schism model) (Wu, Zivanovic, Hayes, & Weiss, 2008).

Recent studies have reported increasing applications of CS NPs directly on food products and packaging materials as an antimicrobial in edible coatings (Pilon, et al., 2015), against cheese microflora (O' Callaghan & Kerry), as a coating on Pacific whiteleg shrimp (Y. B. Wang, et al., 2015) and on red meats (Park, Marsh, & Dawson, 2010). Furthermore, due to CS NPs strong chelation ability they have been used as a carrier for both volatile antimicrobials such essential oils or extracts and polyphenols (Feyzioglu & Tornuk, 2016; Madureira, et al., 2015) and in combination with metal ion nanoparticles such as silver and copper (Du, Niu, Xu, Xu, & Fan, 2009; Tran, et al., 2010; Zain, Stapley, & Shama, 2014). These type of composite NPs have been

applied to pork (J. Hu, Wang, Xiao, & Bi, 2015) and silver carp fillets (Zarei, Ramezani, Ein-Tavasoly, & Chadorbaf, 2015) among other foods.

Nonetheless, technical roadblocks for the application of CS NPs in the food industry exist including their economic cost and unknown toxicological effects. Regarding economic cost, bottom-up synthesis is preferential due to the facile and rapid production of sub 100 nm monodisperse CS NPs compared to top-down methods which have a limited particle size reduction and can also damage the structural properties of CS NPs (Ariga, Hill, & Ji, 2007). (De Lima, et al., 2010) evaluated the genotoxicity of CS-PMMA NPs using cytogenetic tests on human lymphocyte culture and the results indicated that CS-PMMA NPs over a particle size of 82 nm and a concentration of 180 mg L⁻¹ were toxic to human lymphocyte cultures. Additionally, (Y. Wang, et al., 2016) investigated the embryonic toxicology of native CS and CS NPs on zebrafish (*Danio rerio*) where they observed that CS NPs with a particle size of 85 nm had less toxicity towards embryonic cells than native CS suggesting that CS NPs has lower toxicity compared to their bulk counterpart (Ariga, Hill, & Ji, 2007; De Lima, et al., 2010; Y.-L. Hu, Qi, Han, Shao, & Gao, 2011; Y. Wang, et al., 2016)

“Parameters such as initial CS or TPP concentration, CS:TPP mass ratio and pH at formation have individually or in combination been shown to affect the particle size and monodispersity of the synthesised CS NPs (Antoniou, et al., 2015; de Pinho Neves, et al., 2014; Fàbregas, et al., 2013). However, to the best of our knowledge the combined effect of CS MW and pH of formation solution for the development of monodisperse CS NPs with a particle size < 100 nm for applications in the food industry have not been studied. Therefore, the objectives of this study were to assess the effects of MW, pH, CS:TPP mass ratio and initial concentration of CS and TPP on the formation of monodisperse CS NPs using either bottom-up or top-down (tip

sonication or Ultra-Turrax™) techniques. The synthesised nanomaterials were subsequently characterised and their antimicrobial activity assessed

2.3 Materials and Methods

2.3.1 Materials

Low molecular weight chitosan (L. MW) (MW; 50 – 190 kDa, $\geq 75\%$ deacetylated), medium molecular weight chitosan (M. MW) (MW; 190-310 kDa, $\geq 75\%$ deacetylated), aqueous acetic acid (HOAc), sodium hydroxide (NaOH) and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Mueller-Hinton Broth (MHB) and Maximum Recovery Diluent (MRD) were purchased from Oxoid (Basingstoke, UK). Reclaimed planar silicon [100] (p-type) substrates with a native oxide layer (2 nm) were used as received (Intel, Leixlip, Ireland). Cuvettes for zeta potential and particle size analysis were purchased from Malvern (Malvern Instruments Ltd, Malvern, UK).

2.3.2 Synthesis of Chitosan Nanoparticles

Bottom-up synthesis of CS NPs was carried out using ionic gelation method as previously described by Calvo *et al.* (Calvo, et al., 1997) with some modifications. Briefly, different mass ratios of CS to TPP, molecular weight, pH and concentrations of both TPP and CS were assessed. Concentrations of 0.1 % w/v low molecular weight (L. MW) and medium molecular weight (M. MW) CS were dissolved in 1 % v/v acetic acid solution (pH 2.8) and in solution of pH 4.6 or 5.5 adjusted using 1 M NaOH, respectively. These solutions were then stirred at 600 rpm on a magnetic stirrer device (MSH-20D, Wise Stir, Korea) until clear homogenous solutions were formed and then left to stir for 1 h. Separately, 0.1 and 0.25 % w/v solutions of TPP were prepared using sterile deionised water and stirred at 600 rpm for 1 h to ensure complete dissolution of TPP into solution. The TPP (0.1 or 0.25%) solution was added to the

CS (L. MW or M. MW at pH 2.8, 4.6 or 5.5) solutions at a rate of 0.1 mL min^{-1} using a programmable peristaltic pump (Dose It P910, Integra Biosciences AG Switzerland) to a final mass ratio of CS:TPP of 3:1 under constant stirring at 800 rpm. When the concentration of CS was increased from 0.1 to 0.5 % w/v; to reduce the overall average particle size external physical forces were used. These were an Ultra-Turrax™ DT-20 Tube with rotor-stator homogeniser at 4600 rpm for 1 min (IKA Werke, Janke & Kunkel GmbH & Co KG, Staufen, Germany) and tip sonication of CS NPs solutions in an ice bath using a 2 mm probe (EpiShear™ Probe Sonicator, Active Motif, UK) at 20 kHz frequency with an amplitude of 80 % for 10 min in 30 s burst with 10 s rests.

2.3.3 Freeze-drying

Fifteen mL of CS NPs solution were synthesised using 0.1 % w/v L. MW CS at pH of 4.6 using a mass ratio 3:1. These parameters were chosen for freeze drying due to the product's small particle sizes and good monodispersity in order to get a direct weight to weight comparison of native CS to CS NPs for an MIC assay. The CS NP solution was poured into polystyrene petri dishes (Starsedt, Germany) and frozen at -80°C in a deep freezer (Sanyo, Japan) for 12 h before freeze-drying (Lyovac GT2, Steris, Hurth, Germany) under reduced pressure ($p < 0.1 \text{ mbar}$) for 72 h. The freeze dried CS NPs were heat sealed in polyamide/polyethylene vacuum bags (Fispak, Dublin, Ireland) and stored at room temperature under darkness until required for FTIR and antimicrobial analysis.

2.3.4 Zetasizer Particle Characterisation

Particle size analysis, polydispersity index (PDI) and zeta potential measurements of CS and CS NPs were carried out using a Malvern Zetasizer Nano Series HT (Malvern, U.K.). CS NPs solutions (section 2.3.2) were loaded into a disposable cell (ZEN0040) and analysis performed at 25°C using a scattering angle of 173° . The material

refractive index and viscosity of the 1 % v/v acetic acid and water dispersant were defined as 1.333 and 0.890 cP, respectively and the material refractive index (CS NPs) set at 1.52 as previously determined using a handheld refractometer (OPTI, Bellingham + Stanley, Farnborough, U.K.). The particle size distribution and PDI was determined using the Mark–Houwink method while the zeta potential was determined using the Smoluchowski model. For the zeta potential analysis a disposable folded capillary tube (DTS1070) was filled with the CS or CS NPs solution and evaluated in automatic mode. Native CS and CS NPs solution were analysed in duplicate with 3 measurements per sample.

2.3.5 Scanning Electron Microscopy, Atomic Force Microscopy and X-ray Diffraction Characterisation

Silicon wafer coupons (2 cm x 2 cm) were ultrasonicated (Cole-Palmer 8891, IL, USA) for 30 min twice in absolute ethanol. Solutions of native CS, 0.1 or 0.5 % w/v CS NPs, prepared as outlined in section 2.3.2, were diluted (1:10) in absolute EtOH and ultrasonicated for 30 min. The homogenised diluted solutions of native CS or CS NPs solutions were spin coated (Specialty Coating Systems, 6800 spin coat series, IN, USA) onto the cleaned Si wafers at 3000 rpm for 30 s and dried under a stream of nitrogen (N₂) gas. Atomic Force Microscope (AFM, Park systems, XE-100, South Korea) imaging was carried out on the spin coated CS NPs Si wafers and scans performed in non-contact mode with high resolution, silicon micro-cantilever tips. Topographic images were recorded at a resonance frequency of 270-300 kHz. For the SEM imaging, CS NPs deposited on Si wafers were gold sputter coated by mounting loaded Si wafers onto an aluminium SEM stub with double sided carbon tape, and sputter coated with a 5 nm layer of gold/palladium (80:20) using a Quorum Q150 RES Sputter Coating System (Quorum Technologies, UK) and images of native CS and CS

NPs spin coated on Si wafers were captured using a scanning electron microscope (SEM, FEI Company, FEG Quanta 6700). The SEM imaging was carried out at an accelerating voltage of 5 kV. Powder X-ray diffraction (PXRD) patterns of native CS and freeze-dried CS NPs were recorded on a Phillips Xpert PW3719 (Eindhoven, Netherlands) diffractometer using Cu K α radiation. (Cu K α , $\lambda = 0.15418$ nm, operation voltage 40 kV, current 40 mA).

2.3.6 FTIR analysis

Fourier transform infrared spectroscopy (FTIR) analysis of native CS, TPP and freeze-dried CS NPs was performed on a Varian 660-IR spectrometer (Varian Resolutions, Varian Inc, Victoria, Australia) using a diamond crystal ATR Golden Gate (Specac). Scans were taken with 32 scans at 2 cm⁻¹ resolution in a wavenumber range from 4000 - 500 cm⁻¹.

2.3.7 Antimicrobial Assay

2.3.7.1. Bacteria Strains

In this study, antimicrobial activity of native L. MW and M. MW CS or freeze dried L. MW CS NPs against Gram-positive bacteria (*Staphylococcus aureus* (*S. aureus*) (NCIMB 13062) and *Bacillus cereus* (*B. cereus*) (NCIMB 9373)) and Gram-negative bacteria (*Escherichia coli* (*E. coli*) (NCIMB 11943) and *Pseudomonas fluorescens* (*P. fluorescens*) (NCIMB 9046)) were assessed. Before use, all pure culture bacteria were grown for 18 h at 30 °C (*P. fluorescens* and *B. cereus*) or 37 °C (*S. aureus* and *E. coli*) in Mueller-Hinton broth (MHB) (Oxoid, UK) under constant agitation at 170 rpm on an orbital shaker (Innova 2300, New Brunswick™, Germany).

2.3.7.2 Antimicrobial Activity assay

The antimicrobial activity of native CS or freeze dried CS NPs dissolved in 1 % v/v acetic acid solution and pH adjusted to 4.6 were measured by determining the minimum inhibitory concentration (MIC) against the target microorganisms in a 96 flat bottom well tissue culture microplates (Sarstedt Inc., NC, USA) according to the NCCLS (2000) broth microdilution method as described by Cruz-Romero *et al.* (Cruz-Romero, et al., 2013). Bacterial strains were cultured overnight at the appropriate temperature as outlined in section 2.3.7.1 and adjusted to a final density of 10^5 CFU mL^{-1} using maximum recovery diluent and used as an inoculum within 15 min of preparation. Briefly, 100 μL of double-strength MHB (2XMHB) was added to each well in rows A to F, 200 μL of adjusted bacterial culture suspension was added to row H in columns 1–11 and 200 μL of sterile 2XMHB was added to column 12. In each well of row G, 200 μL of 0.1 % w/v native CS or CS NP solutions were dispensed and a twofold serial dilution was performed by transferring 100 μL of antimicrobial solutions from row G into the corresponding wells of row F through to row B. After mixing, 100 μL of the resultant mixture on row B was discarded. Finally, using a 12-channel electronic pipette (Model EDP3-Plus, Rainin, USA) 15 μL of the tested microorganisms was pipetted from each well in row H into the corresponding wells in row A followed by rows B to G. Positive (Row A) and negative growth controls (Column 12) were included in each assay plate. The inoculated plates were incubated in a wet chamber for 24 h at 30 °C (*P. fluorescens* and *B. cereus*) or 37°C (*E. coli* and *S. aureus*). The lowest concentration showing inhibition of growth was considered to be the MIC for the target microorganisms. The test was repeated in duplicate in two independent experiments.

2.3.8 Statistical Analysis

Statistical analysis was performed using the software STATGRAPHICS® centurion XV (Statpoint, Inc., USA). To assess the effects of pH and MW on the particle size, PDI, zeta potential and MIC a two-way analysis of variance (ANOVA) was carried out. Interaction between factors, treatment means and least significant difference (LSD) values are presented ($P < 0.05$). To assess the effects of chitosan to TPP mass ratio, the effects of physical treatments of CS NPs and dissolution pH of freeze dried CS NPs on the particle size distribution, zeta potential, and polydispersity index, a one-way ANOVA was used. A difference between pairs of means was resolved by means of confidence intervals using Tukey's test. The level of significance was set at $P < 0.05$.

2.4 Results and Discussion

2.4.1 Effect of TPP Concentration, Mass Ratio, pH and Molecular Weight on the Formation of Monodisperse Self-assembled Chitosan Nanoparticles

The effects of the initial pH and MW of CS on the average particle diameter, polydispersity index (PDI) and zeta potential of CS NPs are shown in Table 2.1. Both parameters significantly ($P < 0.05$) affected the particle size, PDI and zeta potential of the synthesised CS NPs. Overall L. MW CS produced smaller average particle size compared to M. MW CS. These results are in agreement with the findings of (O' Callaghan, et al., 2016) who reported that the particle size of CS NPs was significantly affected by the MW of CS. However, the relatively small increase in particle size observed between L.MW CS and M.MW CS at pH 4.6 is from the conformation of CS in solution as L. MW CS has an extended rod conformation whereas M .MW has a random coil conformation (Qun & Ajun, 2006). Results of the PDI, a dimensionless measure of the range of the particle size distribution calculated from the cumulant

analysis, indicated that MW significantly ($P < 0.05$) affected PDI of CS NPs where L. MW CS had lower PDI compared to M. MW CS. MW was also shown to have an effect on zeta potential where L. MW CS NPs had a larger overall zeta potential compared to M. MW CS NPs. Regarding the effects of pH on the synthesis of CS NPs, the smallest CS NPs particle size was obtained when CS NPs were synthesised at pH 4.6 while CS NPs synthesised at pH 2.8 gave the largest particle size. These results are in agreement with other studies which have observed that CS NP formed above or below a critical pH range of 4.5 – 5.2 will have larger average particle sizes (Fan, Yan, Xu, & Ni, 2012; Gokce, et al., 2014). The effect of pH on the particle size of CS NPs is believed to be due to the degree of protonation of the CS amine group. At pH 4.6, more protonation of the CS amine occurs which allows for greater interaction with anionic TPP ions, resulting in smaller CS NPs. However, CS NPs synthesised using pH 2.8 showed larger particle diameter and may perhaps be due to the increased ionic strength of CH_3CHOO^- anions shielding interaction of protonated CS amine groups with anionic TPP, therefore, reducing the number of crosslinking sites and resulting in larger CS NPs (Fan, et al., 2012). At pH 5.5, CS amine is less protonated and consequently more anionic TPP has to interact with the CS polymer to stabilise the CS NPs and so resulting in larger CS NPs (Mi, Shyu, Lee, & Wong, 1999). With respect to PDI, CS NPs at pH 5.5 had the lowest PDI while NPs synthesised using pH 2.8 had the largest PDI. The pH was also shown to affect zeta potentials as the zeta potential (ZP) at pH 2.8 the largest ZP value was measured whilst at pH 5.5 the lowest zeta potential was recorded. This may be linked to the degree of protonation of the CS NPs solution.

The interactive effect of MW and pH was also shown to have a significant ($P < 0.05$) effect on the particle size distribution, PDI and zeta potential of the synthesised CS

NPs. The lowest particle size and PDI was observed when CS NPs were synthesised using L. MW CS at pH 4.6 (92 nm) and the largest CS NPs particle size and PDI was observed using M. MW CS at pH 2.8. Furthermore, zeta potential measurements show that L. MW and M. MW CS NPs synthesised at pH 2.8 had the largest zeta potentials (Table 2.1). Additionally, the zeta potential of L. MW CS NPs at pH 4.6 was recorded to be also above the ± 30 mV threshold and are considered as moderately stable colloids and are therefore, less susceptible to agglomeration and destabilisation forces such as van der Waals forces, Brownian motion or particle – particle interactions (Gokce, et al., 2014; Mohanraj VJ, 2006).

Since L. MW CS at pH 4.6 gave CS NPs with the smallest average particle diameter and more stable colloids (due to the higher zeta potential), the effect of TPP concentration and mass ratio of CS:TPP on the synthesis of CS NPs was investigated using this reaction condition. Initially, the TPP concentration was increased from 0.1 to 0.25 % w/v maintaining a constant mass ratio of CS:TPP where the particle size analysis indicated that the initial concentration of TPP significantly ($P < 0.05$) affected the particle size of the formed CS NPs, with greater particle size and higher PDI obtained in CS NPs synthesised using 0.25 % w/v TPP (Table 2.2). These results are in agreement with the findings of (Fan, et al., 2012) who reported that the reduction of TPP concentration corresponded to a reduction in the overall particle size distribution of CS NPs; whereas higher concentrations of TPP result in larger, polydisperse CS NPs (Qi, Xu, Jiang, Hu, & Zou, 2004). The use of concentrations of 0.1 % w/v TPP yielded smaller NPs due to the smaller number of counter ionic sites of TPP interacted with CS; reducing the potential for an excess net negative charge from the TPP needing to be balanced with more cationic CS, which can result in the agglomeration of CS NPs. The CS:TPP mass ratio affected the formation of CS NPs

Table 2.1 Effects of pH and chitosan molecular weight on the particle size distribution, polydispersity index and zeta potential during synthesis of chitosan nanoparticles synthesised from 0.1 % w/v low or medium molecular weight chitosan at pH 2.8, 4.6, or 5.5 using 3:1 CS:TPP mass ratios.*

	Particle size distribution (nm)	PDI	Zeta potential (mV)
A: MW			
L.MW	120.057 ^a	0.266a	29.46 ^a
M.MW	186.006 ^b	0.481 ^b	26.21 ^b
B: pH			
pH2.8	224.042 ^a	0.679 ^a	44.14 ^a
pH4.6	104.793 ^b	0.235 ^b	25.96 ^b
pH5.5	130.258 ^c	0.207 ^c	13.39 ^c
Interaction (A × B)			
L.MW,pH2.8	143.07 ± 2.46 ^a	0.462 ± 0.007 ^a	42.28 ± 186 ^a
L.MW,pH4.6	92.92 ± 6.69 ^b	0.155 ± 0.038 ^b	30.15 ± 2.45 ^b
L.MW,pH5.5	120.58 ± 10.82 ^c	0.173 ± 0.017 ^c	15.93 ± 1.20 ^c
M.MW,pH2.8	305.02 ± 27.82 ^d	0.896 ± 0.036 ^d	46.00 ± 1.52 ^d
M.MW,pH4.6	113.07 ± 8.09 ^e	0.307 ± 0.067 ^e	21.80 ± 1.98 ^e
M.MW,pH5.5	139.93 ± 9.09 ^f	0.242 ± 0.049 ^f	10.84 ± 0.77 ^f
LSD	3.122	0.0096	0.405

*All values are means of triplicate measurements from two independent experiments (n=6)

^{a,b,c,d,e,f} different subscripts in the same column indicate significant differences ($P < 0.05$).

in a complex manner. The 6:1, 3:1 and 1:1 CS:TPP mass ratios (maintaining a constant CS concentration of 0.1 % w/v (Table 2.2)) indicated that the most stable CS:TPP mass ratio for the formation of CS NPs was 3:1 since CS NPs formed using this mass ratio had the smallest average particle diameter (90.5 nm) and PDI (0.164) while having the highest zeta potential (30.15 mV). This represents the basic CS NP colloid unit and when parameters (such as mass ratio or concentration) are changed, aggregation of CS NPs into larger agglomerates occurs. Comparatively, mass ratios of 6:1 and 1:1 showed significantly ($P < 0.05$) larger PDI values and lower zeta potential measurement (Table 2.2) while 1:1 samples also had significantly ($P < 0.05$) larger particle size distribution than 3:1 and 6:1 samples. A study carried out by Antoniou *et al.* (Antoniou, et al., 2015) used concentrations of 0.5 mg mL⁻¹ L. MW CS and 0.7 mg mL⁻¹ TPP in different mass ratios to synthesise CS NPs and found that the CS NPs particle size distribution decreased when the mass ratio CS:TPP up to 9:1 was used; however, when larger concentration of CS than 9:1 mass ratios were used, larger CS NPs were formed.

In this study, CS NP solutions made using a mass ratio of 6:1 were transparent while solutions made using a 3:1 mass ratio were slightly opaque; however, solutions with a mass ratio of 1:1 were turbid and large agglomerates were observed (Figure 2.2). Since CS NPs are polymers, their nucleation mechanism of formation will differ if compared to metallic ion nanoparticles. (Rathi & Gaikar, 2017) reported that higher CS concentration leads to the generation of many nuclei; however, these nuclei have higher growth rates due to repulsion between the CS chains and result in the formation of bigger CS NPs.

Table 2.2 Effects of chitosan to TPP mass ratio on the particle size distribution, polydispersity index and zeta potential at formation of chitosan nanoparticle synthesised from 0.1 % w/v low molecular weight chitosan at pH 4.6 using 6:1, 3:1 and 1:1 CS:TPP mass ratios

	Particle size distribution (nm)	PDI	Zeta potential (mV)
0.1 % w/v L. MW CS NPs (6:1)	100.17 ± 0.89 ^a	0.264 ± 0.016 ^a	20.48 ± 1.68 ^a
0.1 % w/v L. MW CS NPs (3:1)	96.52 ± 2.83 ^a	0.195 ± 0.013 ^{a,b}	30.15 ± 2.45 ^b
0.1 % w/v L. MW CS NPs (1:1)	20206.67 ± 4651.12 ^b	0.458 ± 0.287 ^b	9.81 ± 1.43 ^c

*All values are means of triplicate measurements from two independent experiments (n=6)

^{a,b,c}, Mean values in the same row with different superscripts are significantly different ($P < 0.05$).

To assess the effects of initial CS concentration at formation of CS NPs at pH 4.6; initial concentration of L. MW CS was increased from 0.1 to 0.5 % w/v (Table 2.3). Results showed that the increase of the initial concentration significantly increased ($P < 0.05$) the average particle diameter of CS NPs from 90.5 nm to 1687.167 nm before physical treatments and increased the PDI of CS NPs from 0.195 to 0.662 (Table 2.3). Therefore, size reduction techniques such as tip sonication and an Ultra-Turrax™ DT-20 were employed to reduce the particle size of the agglomerated CS NPs. While both treatments showed a significant ($P < 0.05$) reduction in the average particle size and PDI compared to untreated CS NPs, size reduction was more efficient ($P < 0.05$) when tip sonication was used compared to Ultra-Turrax™ DT-20 Tube (Table 2.3);



Figure 2.2 Visual Image of chitosan nanoparticle synthesised from 0.1 % low molecular weight chitosan at pH 4.6 using 6:1, 3:1 and 1:1 CS:TPP mass ratios (from left to right) where 1:1 is the most turbid while 3:1 is least turbid.

however, Ultra-Turrax™ DT-20 treatment showed a significant increase in the zeta potential compared to untreated and tip sonicated CS NPs. Previous studies have reported that tip sonication treatment is more effective at size reduction of CS NPs due to its mechanism of particle breakdown and is through a cavitation mechanism whereby acoustic energy generated by the sonicator creates rapidly collapsing bubbles thus creating a transient high pressure gradient and high velocity within the liquid which subsequently creates a shear force that can break the CS polymer chain at the $\beta(1-4)$ linkage, due to the difference in bond energy between the two moieties (Tao Wu, 2008). Furthermore, other breakdown mechanism such as hydrolysis or fragmentation mechanisms have also been reported (Czechowska-Biskup, Rokita, Lotfy, Ulanski, & Rosiak, 2005). Moreover, tip sonication is also advantageous as its particle size reduction mechanisms precludes CS NPs below a certain particle size, reducing potential damage to their structure and targeting larger CS NPs for size reduction (Antoniou, et al., 2015).

Table 2.3 Effect of physical treatments on the particle size distribution, zeta potential, and polydispersity index of chitosan nanoparticle synthesised using 0.5 % w/v low molecular weight chitosan, 3:1 CS:TPP ratio and pH 4.6 *

	Particle size distribution (nm)	PDI	Zeta potential (mV)
0.5 % w/v CS NPs untreated	1687.167 ± 412.27 ^a	0.662 ± 0.114 ^a	31.32 ± 3.49 ^a
0.5 % w/v CS NPs Ultra-Turrax™	1130.83 ± 98.01 ^b	0.446 ± 0.157 ^b	44.75 ± 2.76 ^b
0.5 % w/v CS NPs Tip sonication	385.13 ± 10.44 ^c	0.303 ± 0.034 ^c	32.35 ± 3.79 ^a

*All values are means of triplicate measurements from two independent experiments (n=6)

^{a,b,c}. Mean values in the same row with different superscripts are significantly different ($P < 0.05$).

When freeze-dried (0.1 % w/v) CS NPs were dispersed in water or 1 % v/v HOAc (pH 2.8) or pH 4.6 (adjusted using 1 M NaOH) and tip sonicated to deagglomerate CS NPs into the solution; the largest particle size was observed when CS NPs were dispersed in water (1288 nm) while the smallest average particle diameter was observed when CS NPs were dispersed in 1% HOAc at pH 4.6 solution (318 nm). Similarly, the smallest PDI value was recorded for CS NPs at pH 4.6 whereas CS NPs dispersed in pH 2.8 and 7 had larger PDI values. The results indicated that freeze-dried CS NPs were significantly larger than CS NPs synthesised in situ: however, the zeta potential followed a similar trend that linearly increased when pH decreased (Table 2.4).

Table 2.4 Effect of tip sonication treatment on the particle size distribution, polydispersity index and zeta potential of freeze-dried of chitosan nanoparticles synthesised from 0.1 % w/v low molecular weight chitosan at pH 4.6 using a 3:1 CS:TPP mass ratios dissolved at pH 7, 4.6 or 3.*

	Particle Size		Zeta Potential (mV)
	Distribution (nm)	PDI	
0.1 % w/v CS NPs (Freeze-dried, pH 7)	1287.92 ± 424.55 ^a	0.855 ± 0.109 ^a	13.07 ± 12.87 ^a
0.1 % w/v CS NPs (Freeze-dried, pH 4.6)	317.55 ± 71.08 ^b	0.350 ± 0.045 ^b	22.68 ± 1.79 ^b
0.1 % w/v CS NPs (Freeze-dried, pH 3)	677.27 ± 109.08 ^c	0.693 ± 0.052 ^c	39.73 ± 0.98 ^c

*All values are means of triplicate measurements from two independent experiments (n=6)

^{a,b,c}, Mean values in the same row with different superscripts are significantly different ($P < 0.05$).

2.4.2 Morphological and Topographical Analysis of Chitosan Nanoparticles

The morphological features of native L. MW CS and CS NPs synthesised using bottom-up and top- down techniques are shown in Figure 2.3 while topographical features of 0.1 and 0.5 % w/v L. MW CS NPs made using L. MW CS at pH 4.6 are shown in Figure 2.4. Native L. MW CS at an initial concentration of 0.1 % w/v has semi-crystalline nature with a fibril and irregular morphology and a wide particle size distribution (Figure 2.3.a). CS NPs synthesised through a bottom-up self-assembly

process using an initial concentration of 0.1 w/v L. MW CS at pH 4.6 using a 3:1 CS to 0.1 % w/v TPP mass ratio had a regular spherical morphology and a monodisperse particle size distribution typical of rapid solution processing (Figure 2.3.b). However, when the initial concentration of CS was increased to 0.5 % w/v agglomeration of CS NPs was observed (Figure 2.3.c). Therefore, tip sonication was applied to CS NPs and resulted in NP with rod-like morphologies and a significant particle size reduction and an increase in monodispersity (Figure 2.3.d). Furthermore, CS NPs synthesised using 0.5 % w/v CS also showed larger average particle diameter

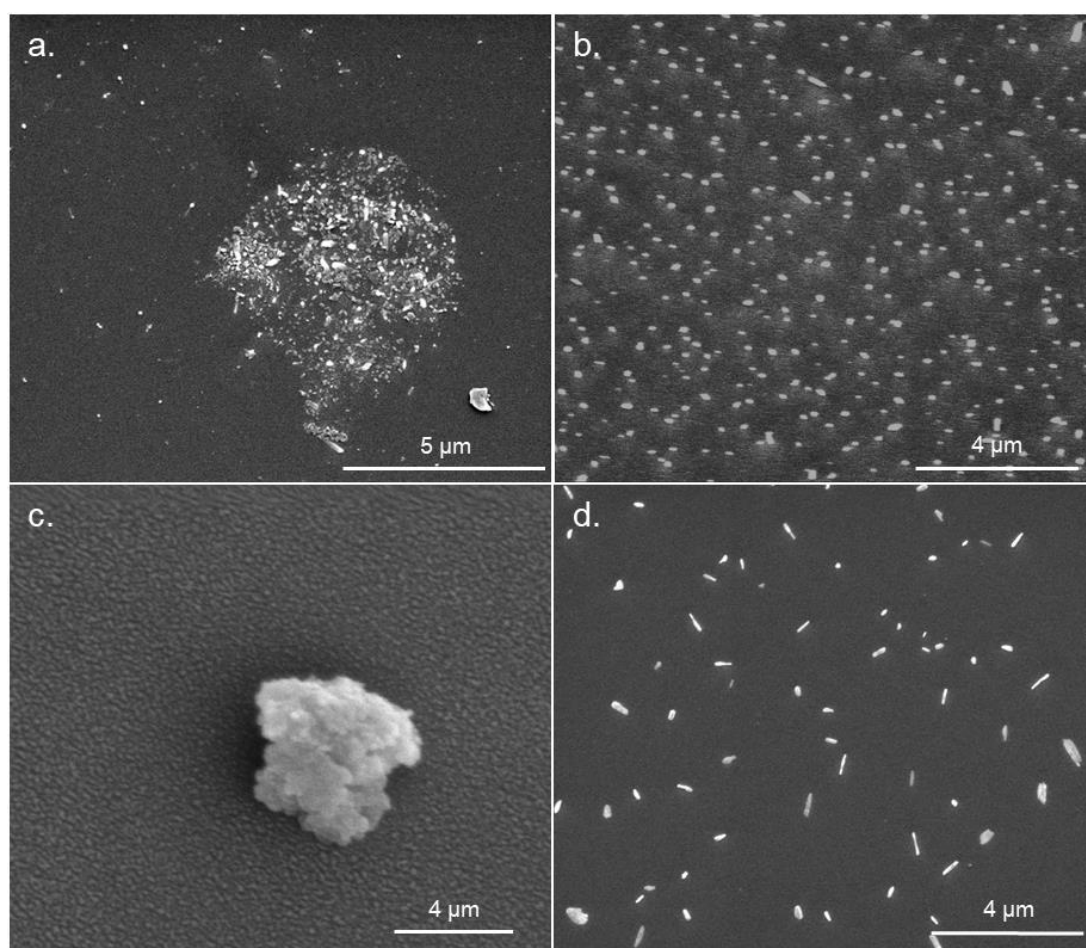


Figure 2.3 SEM micrograph of native low molecular weight chitosan (a), 0.1 % w/v chitosan nanoparticles (b), untreated 0.5 % w/v chitosan nanoparticles (c) and tip sonicated treated 0.5 % w/v chitosan nanoparticles. Chitosan nanoparticles were synthesised from low molecular weight chitosan at pH 4.6 using a 3:1 CS:TPP mass ratio.

compared to NPs synthesised using 0.1 % w/v CS. For AFM analysis, due to the irregular and large size features of native CS, topographical imaging was carried out only on 0.1 and 0.5 % w/v CS NPs and the results showed that the average particle diameter of CS NPs were 26.59 and 34.18 nm, respectively (Figure 2.4). The particle size obtained using AFM analysis was up to 70.6 or 92.2 % smaller for 0.1 and 0.5 % w/v CS NPs, respectively than the results obtained by the Zetasizer. Due to the hygroscopic nature of the CS NPs, apparently the drying effect of absolute EtOH used as dispersant of the CS NPs significantly ($P < 0.05$) reduced overall particle size obtained using the AFM compared to the particle size obtained using the hydrodynamic analysis technique. This results suggests that CS NPs are extremely

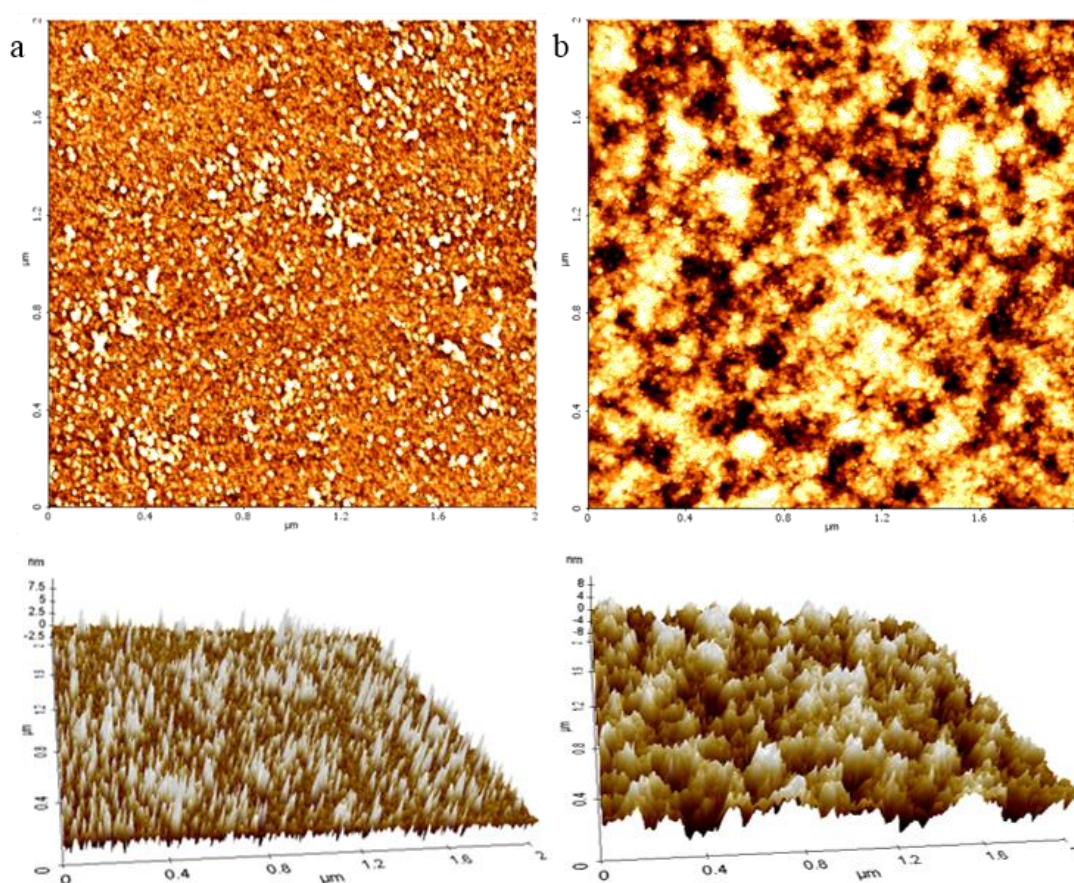


Figure 2.4 AFM images ($2 \times 2 \mu\text{m}$) of 0.1 (a) and 0.5 (b) w/v% chitosan nanoparticles synthesised from low molecular weight chitosan at pH 4.6 using a 3:1 CS:TPP mass ratio showing both 2D and 3D topographical images

swollen in aqueous solution and have good absorption ability which can be used to enhance the antimicrobial activity of the CS NPs as studies have showed that the incorporation of other NAMs such as essential oils or non-NAMs such as silver nanoparticles, can increase the antimicrobial activity of CS NPs (Hosseini, Rezaei, Zandi, & Farahmandghavi, 2016; Zain, et al., 2014). Typical PXRD diffractograms showed a low intensity peak at 20.13° and this results are in agreement with results reported by (Shahbazi, Rajabzadeh, & Ahmadi, 2017). However, the PXRD analysis of CS NPs did not show a distinct peak indicating the amorphous nature of CS NPs which was attributed to the crosslinking between CS & TPP which prevents alignment of the chitosan chains (Figure 2.5)(Qi, et al., 2004; Shahbazi, et al., 2017).

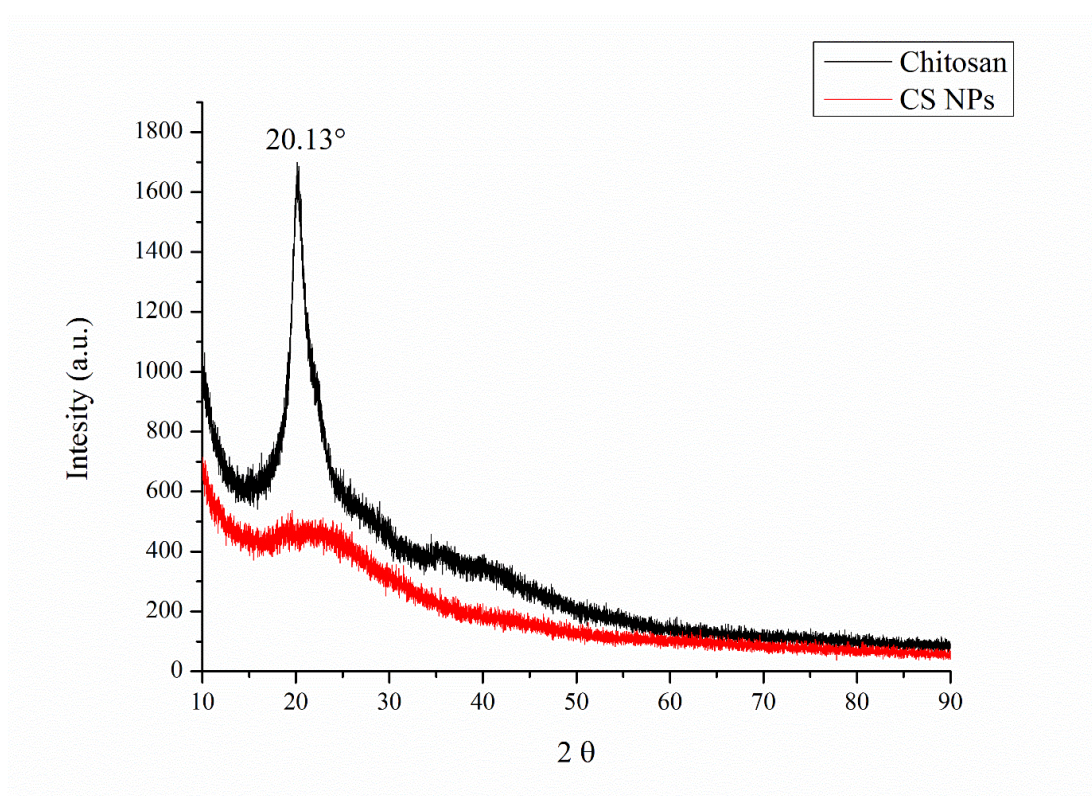


Figure 2.5 PXRD spectra of chitosan (—) and chitosan nanoparticles (—) synthesised from 0.1 % w/v low molecular weight chitosan at pH 4.6 using a 3:1 CS:TPP mass ratio

2.4.3 FTIR Analysis of Chitosan and Chitosan Nanoparticles

The FTIR spectrum of native L. MW CS, TPP and freeze dried L. MW CS NPs are shown in Figure 2.6. The FTIR spectra of native L. MW CS showed major peaks at: 1027 cm^{-1} indicating a C – O – C symmetric stretch (from the glucosamine unit of CS), 1542 cm^{-1} which suggests an aromatic C – C bonds, 1650 cm^{-1} indicated a bending 1° amine, 2865 cm^{-1} suggests a C – H vibrational stretching band and the broad peak from $3200 - 3500\text{ cm}^{-1}$ is a combination of symmetric and asymmetric –NH₂ and vibrational –OH stretches and these results are in agreement the result reported by (de Pinho Neves, et al., 2014). The CS NP spectra differed from the native CS where several peaks such as the aromatic C – C which shifted from 1585 cm^{-1} to 1542 cm^{-1} and the primary amine at 1650 cm^{-1} shifted to 1633 cm^{-1} while the broad

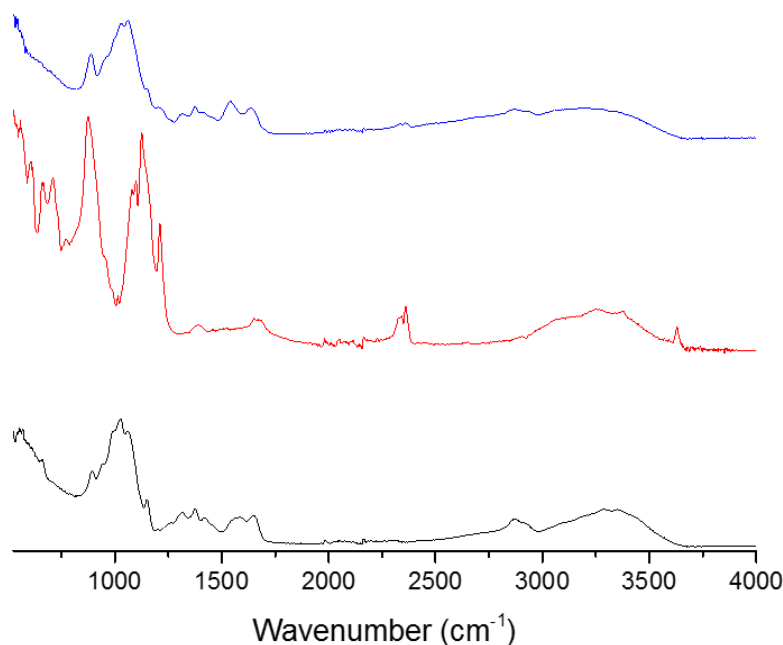


Figure 2.6 FTIR spectra of chitosan (—), sodium tripolyphosphate (—) and chitosan nanoparticles (—) synthesised from 0.1 % w/v low molecular weight chitosan at pH 4.6 using a 3:1 CS:TPP mass ratio

overlapping N – H and O – H peak at $3200 - 3400\text{ cm}^{-1}$ has decreased peak intensity and definition suggesting that ionic gelation occurred between the protonated amine and the counter anion present in TPP. However, the C – O – C peak at 1027 cm^{-1} and the C – H peak at 2865 cm^{-1} remained unaffected and this results are in agreement with the results reported by (Antoniou, et al., 2015) and (de Pinho Neves, et al., 2014). The FTIR spectra of TPP powder showed peaks at 875 cm^{-1} corresponding to both O – P – O and P – O stretches and a peak at 1126 cm^{-1} which corresponded to a P = O stretch. The peak intensity at those wavelengths were lower in the CS NP spectra and this results are similar to the reported by (Siripatrawan & Harte, 2010).

2.4.4 Antimicrobial Activity of Chitosan and Chitosan Nanoparticles

Antimicrobial activity of L. MW and M. MW CS at pH 2.8, pH 4.6 or pH 5.5, respectively and freeze-dried CS NPs synthesised at pH 4.6 using a mass ratio of 3:1 CS:TPP against Gram-positive (*S. aureus* and *B. cereus*) and Gram-negative (*E. coli* and *P. fluorescens*) bacteria (common food spoilage microorganisms) indicated that the pH and MW of native CS significantly ($P < 0.05$) affected the antimicrobial efficacy (Table 2.1). Independent of pH, L. MW CS was observed to have greater antimicrobial activity against *S. aureus*, *B. cereus* and *P. fluorescens*; however, *E. coli* was shown to be more susceptible to M. MW CS. Studies in the literature have widely reported that L. MW CS has better antimicrobial activity, due its ability to penetrate the cell wall of microbes and combine with their DNA, thus inhibiting the synthesis of mRNA and DNA transcription (Cruz-Romero, et al., 2013; Liu, et al., 2006). However, other authors have reported that M. MW CS is more effective as it can form a film that encapsulates bacteria, inhibiting nutrient uptake (Chang, Lin, Wu, & Tsai, 2015). This suggests that the primary mechanism of antimicrobial activity of CS differed between bacterial strains. Independent of MW, pH was shown to have a

significant effect on the antimicrobial activity. CS at pH 2.8 was the most effective antimicrobial while CS at pH 5.5 had the least effective antimicrobial activity. Herein it has been shown that lower pH (2.8) increased the antimicrobial activity. Similarly, previous studies have found that significantly higher antimicrobial activity of CS occurs at lower pH compared to a higher pH as a result of increased CS amine group protonation in acidic solutions (Kong, et al., 2010; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Overall it was observed that the greatest antimicrobial activity of native CS was L. MW CS at pH 2.8, while the least antimicrobial effectiveness was observed using L. MW or M. MW CS at pH 5.5.

The antimicrobial activity of the synthesised CS NPs indicated that *E. coli* and *B. cereus* were less susceptible to the CS NPs than *P. fluorescens* and *S. aureus* (Figure 2.7). Herein it was observed that the MIC of CS NPs against *P. fluorescens* and *S. aureus* was 0.125 mg mL⁻¹ while a concentration of 0.25 mg mL⁻¹ was needed to inhibit *E. coli* and *B. cereus*. Comparatively, MICs values obtained in this study are lower than the MIC against *S. aureus*, *B. cereus*, *P. fluorescens* and *E. coli* reported by O' Callaghan *et al.* (O' Callaghan, et al., 2016) who found the most susceptible bacteria to CS NPs was *P. fluorescens* (0.22 mg mL⁻¹) while the least susceptible bacteria was *S. aureus* (0.28 mg mL⁻¹). In this study, CS NPs did not show enhanced antimicrobial activity when compared to native CS. Our results are in agreement with the findings of Ristić *et al.* who reported that CS had greater antimicrobial activity than CS NPs due to the cationic amines being taken by the ionic gelation and resulted in less protonated amines available for interaction with negatively charged components of bacterial cells (Ristić, Lasič, Kosalec, Bračić, & Fras-Zemljič, 2015). This may also be due to the strong solvent swelling character of CS NPs, as when in aqueous solution the particle size of CS NPs will increase and this may perhaps reduce

the potential ability to interact with bacterial cell component reducing the antimicrobial activity of the CS NPs. Furthermore, when CS and CS NPs were compared on a percent weight by weight basis (% w/w), the concentration of CS in CS NPs was lower due to a different structural composition of CS NPs and native CS as CS NPs have in their structure antimicrobial CS and TPP as they were synthesised using a 3:1 (CS:TPP) mass ratio which may decrease the antimicrobial activity of CS NPs if compared to a similar weight of native CS. In addition the different structural composition of CS NPs may perhaps explain the reduced toxicity of CS NPs as (Y. Wang, et al., 2016) found that CS NPs were less toxic than native CS and as reported herein native CS and CS NPs have the similar antimicrobial activity which may apparently make the application of CS NPs in food more favourable compared to CS (Y. Wang, et al., 2016). Nevertheless, studies have reported that CS NPs showed enhanced antimicrobial properties compared to their non-nano equivalents (Ngan, et al., 2014; Qi, et al., 2004). Enhanced antimicrobial activity of CS NPs was attributed to the increased surface area compared to their bulk counterpart thus allowing more interaction with the bacterial cell components (Maillard & Hartemann, 2013).

Additionally, it has been reported that the antimicrobial activity of native CS is not only affected by pH and MW but also by other intrinsic and extrinsic factors such as degree of deacetylation (DD) of CS and bacterial type (e.g. Gram stain). The effects of the DD on the antimicrobial activity of CS were reported to be due to the greater number of amine groups that can be protonated (Li, Wu, & Zhao, 2016). In general an increased DD was reported to have an increased antimicrobial activity; however, when DD > 85 %, antimicrobial efficacy of CS does not significantly increase (Li, et al., 2016). The Gram strain of the bacteria has also been reported to affect CS antimicrobial efficacy. Herein, within the Gram-negative bacteria tested *P. fluorescens*

was the most susceptible to native CS compared to *E. coli*. Concerning Gram-positive bacteria, it was found that *B. cereus* was more susceptible to native CS than *S. aureus*. It has been reported that Gram-negative bacteria such as *Pseudomonas* spp. are more susceptible due to polycationic CS that can compete with stabilising divalent metals (e.g. Mg^{2+} , Ca^{2+}) in the cell wall of Gram-negative bacteria leading to structural destabilisation (Cruz-Romero, et al., 2013). However, other studies have reported that CS is more antimicrobially active against Gram-positive bacteria such as *S. aureus* due to CS ability to form linkages with lipoteichoic acid on the cell surface of Gram-positive bacteria, disrupting membrane functions (Goy, Morais, & Assis, 2016). Regarding CS NPs, Gram-positive bacteria such as *B. cereus* may show less susceptibility owing to the positive zeta potential of CS NPs hindering interaction with

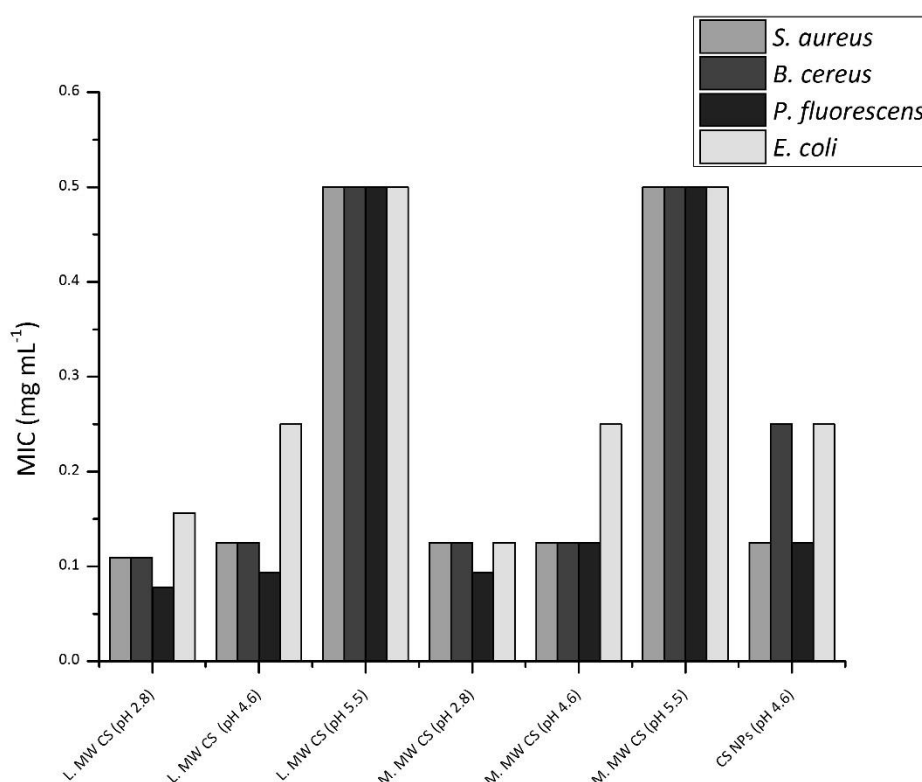


Figure 2.7 Effects of pH (2.8, 4.6 or 5.5) or molecular weight (low or medium molecular weight) of chitosan and low molecular weight chitosan nanoparticles at pH 4.6 on the antimicrobial activity (Minimum inhibition concentration ($mg\ mL^{-1}$)).

bacterial cells (Simon-Deckers, et al., 2009). Furthermore, *E. coli* may showed less susceptibility towards L. MW CS NPs compared to M. MW CS.

2.5 Conclusions

Factors such as MW, pH, CS and TPP concentration and CS:TPP mass ratio significantly affected ($P<0.05$) the particle size of the synthesised CS NPs. The interactive effect of MW and pH on the formation of CS NPs showed that optimal particle size was obtained when L. MW CS at a pH of 4.6 was used. The parameters to synthesise monodisperse CS NPs using the bottom-up technique with an average particle size of 90 nm and zeta potential of 30.15 mV were 0.1 % w/v L. MW CS at pH 4.6 and 3:1 (CS:TPP) mass ratio. When higher initial concentrations of CS were used, the application of external forces such as tip sonication was required to induce monodispersity and reduce the particle size; however, the particle size was > 300 nm. In native CS the initial pH significantly affected ($P<0.05$) the antimicrobial activity where pH 2.8 showed greater antimicrobial efficacy compared to CS solutions at pH 4.6 and 5.5. While antimicrobial activity of CS NPs was not significantly higher than native CS; however, monodisperse CS NPs unique physicochemical properties may find applications in the development of antimicrobial active packaging.

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**Chapter 3: A Novel Method to Deliver Natural Antimicrobial Coating Materials
to Extend the Shelf-life of European Hake (*Merluccius Merluccius*) Fillets**

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Joseph P. Kerry, Michael A. Morris

This Chapter has been submitted for publication in “Food Packaging and Shelf-life”.

3.1 Abstract

The objectives of this study were to investigate the effectiveness of aerosolisation as a novel coating method of nano and non-nano natural antimicrobials solutions of chitosan (CS), chitosan nanoparticles (CS NP) and commercially available carnosolic acid nano-solubilise (CASB) as an additional hurdle to extend the shelf-life of vacuum skin packaging (VSP) European Hake (*Merluccius merluccius*) fillets caught off the Irish sea. The aerosolisation of hake fillets with natural antimicrobial solutions of 0.1 % w/v CS, 0.1 % w/v CS NP, or 0.8 % w/v CASB extended significantly ($P<0.05$) the shelf-life of VSP hake fillets by up to 40, 50 and 55 %, respectively compared to untreated control samples. The pH of hake fillets was significantly increased by CS and CS NP treatments compared to untreated control and CASB treatments. The colour parameters of treated hake fillets increased significantly throughout storage, independent of treatment; however, lightness was significantly ($P<0.05$) reduced by CASB treatments, redness was significantly ($P<0.05$) increased by CS NP treatments, and yellowness was significantly ($P<0.05$) increased with all CS, CS NP, and CASB treatments. In addition, the whiteness index (WI) of hake fillets was reduced significantly ($P<0.05$) when aerolised with CS, CS NP, and CASB treatments. The lipid oxidation (TBARS) of hake fillets, independent of the antimicrobial treatment, decreased significantly ($P<0.05$) throughout storage. The results from this study indicates that aerosolisation of natural antimicrobial materials can effectively extend the shelf-life of fish products.

3.2 Introduction

Hake fillets (*Merluccius merluccius*) are a prime fish product, predominantly consumed in European markets. However, hake, like many other fish products, are

highly perishable and deteriorate rapidly post mortem due to various biochemical and microbial breakdown mechanisms as a result of their high water content (65 – 80 %), mild pH of 6 - 7 and the presence of large quantities of non-protein nitrogen (9 - 18 %). In addition, environmental factors such as catching region and season, and their handling post mortem all contribute to the short shelf-life of hake fillets (Fernandez-Saiz, Sanchez, Soler, Lagaron, & Ocio, 2013; Garcia-Soto, Aubourg, Calo-Mata, & Barros-Velazquez, 2013; Otero, Perez-Mateos, & Lopez-Caballero, 2017). Moreover, it is estimated that between 30 – 50 % of harvested fish are lost in the supply chain (Garcia, et al., 2015).

Typically fish products intended for consumers are packaged using technologies such as modified atmosphere packaging (MAP), vacuum packing (VP) and vacuum-skin packaging (VSP). It was reported that compared to aerobically stored hake, these packaging technologies have significantly extended the shelf-life of hake fillets (Carrion-Granda, Fernandez-Pan, Rovira, & Mate, 2018; Erkan, 2012). In addition, further shelf-life extension can be achieved through the application of hurdle technology, which is an intelligent combination of mild preservation technologies (Kerry, 2012). One such hurdle is the application of natural antimicrobial materials (NAM's) such as chitosan (CS) and essential oil (EO) based materials. The application of these materials are favoured due to their properties such as “GRAS” status, biodegradability, biocompatibility, and antimicrobial properties against a wide range of spoilage and pathogenic microorganisms (D. J. Sullivan, et al., 2018). Currently, NAM's materials are applied to food products as part of the hurdle approach, through various technologies including: direct incorporation into the polymeric packaging material (Yang, et al., 2016), coating onto packaging materials surface (Erkan, 2012), and using an inherently antimicrobial film forming polymer (such as CS) (Fernandez-

Saiz, et al., 2013) or a combination thereof (Shahbazi & Shavisi, 2018). To extend the shelf-life of food products, NAM's have been applied to various foods such as tomatoes (Tripathi, Mehrotra, & Dutta, 2009), chicken breasts (Latou, Mexis, Badeka, Kontakos, & Kontominas, 2014), sea bass fillets (Günlü & Koyun, 2013), and ready-to-eat (RTE) shrimp (Guo, Jin, Scullen, & Sommers, 2013), oysters (Rong, Qi, Yin, & Zhu, 2010), salmon (Souza, et al., 2010), silver carp (Abdollahi, Rezaei, & Farzi, 2014), cod fillets (Gomez-Estaca, Lopez de Lacey, Lopez-Caballero, Gomez-Guillen, & Montero, 2010), whiteleg shrimp (Y. B. Wang, et al., 2015), and beef (Vilela, et al., 2016).

Aerosolisation, defined as the dispersion of a liquid phase into air in the form of fine mist, is usually used for sanitary purposes, especially for respiratory medical treatments and room disinfection (Andersen, et al., 2006). However, to the best of our knowledge the application of aerosolised NAM's to fish products as a novel method to deliver antimicrobial coating and enhance microbiological quality and shelf life extension of these products has not been investigated. This type of delivery offers a number of advantages including relatively ease of application and higher surface coverage and penetration due to the small droplet compared to conventional spray coating size. However, roadblocks include the scaling up of the aerosolisation unit for commercial application and the limited number of antimicrobials that can be aerosolised (Jiang, et al., 2017). Nonetheless, nebulizers are a readily available apparatus that are widely used as an aerosolisation system for delivery of drugs into the lungs. This is via the generation of a fine "mist" with a typical average droplet diameter of $\geq 5 \mu\text{m}$. This is a smaller droplet diameter than conventional spray coated generated mist or from a typical electrostatic sprayer (Jiang, et al., 2017; Park, et al., 2012). Currently, most studies involving aerosolisation treatment systems have

focused on organic acid sanitisers and disinfectants such as peroxyacetic acid and sodium hypochlorite due to their water solubility and broad antimicrobial activity and have been applied on to various fruits such as cherry tomatoes (Jiang, et al., 2017) and strawberries (Vardar, Ilhan, & Karabulut, 2012). However, nanotechnology, which is the development and application of materials that have one or more dimensions of the order of 100 nanometres (nm) or less (Chaudhry, et al., 2008), may allow for the increase in scope of materials that could be applied via aerosolisation due to their small size features which could be readily dispersed, either as a particle, colloid or micelle, in an aerosolisable aqueous solution. Accordingly, the use of the nano equivalents of CS and EO's have been widely reported as antimicrobial active agent in food packaging applications (David Joseph Sullivan, et al., 2018), and given their previously outlined properties make them ideally suited to this application.

While studies have reported on the use of aerosolisation of antimicrobials as a treatment method to extend shelf of food products (Jiang, et al., 2017; Vardar, et al., 2012); to the best of our knowledge, none have investigated the use of aerosolisation of nano and non-nano NAM as means to deliver antimicrobial coating as treatment in a hurdle strategy to extend the shelf-life of hake fillets. Therefore, the objective of this study was to evaluate the effects of the aerosolisation of CS, CS NPs and CASB antimicrobial solutions, as an additional hurdle to extend the shelf life of VSP hake fillets.

3.3 Materials and Methods

3.3.1 Materials

Skinned Hake fillets (hitherto referred to as hake fillets) were purchased from Ballycotton Seafood Ltd (Garryvoe, Ireland). Low molecular weight chitosan (L. MW) (MW; 50 – 190 kDa, $\geq 75\%$ deacetylated), aqueous acetic acid (HOAc), sodium

hydroxide (NaOH) and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. NovaSOL® Rosemary, a commercially available novel water and fat soluble 6% carnosolic acid nano-solubilisate (CASB) was obtained from AQUANOVA® (AQUANOVA AG, Darmstadt, Germany). Media for microbiological analysis including: plate count agar, De Man, Rogosa and Sharpe (MRS) agar and *Pseudomonas* Agar Base with selective supplement CFC (cetrimide, fucidin, cephaloridine) (SR0103) were purchased from Oxoid, Tryptic Soy Agar was purchased from Merck and Compact Dry-EC chromogenic plates were obtained from Nissui Pharmaceutical (Co. Ltd. Japan). Lyngby agar was made using (g L⁻¹): peptone (20), bacteriological agar (15), sodium chloride (5), sodium thiosulfate (0.3), yeast extract (3), beef extract (3), L-cysteine (0.6) and ferric citrate (0.3) which were all purchased from Sigma Aldrich.

3.3.2 Preparation and Aerosolisation of Antimicrobials onto Hake Fillets

3.3.2.1 Preparation of Natural Antimicrobial Solutions

Solutions of 0.1 % L. MW CS and CS NP were prepared as previously outlined by (D. J. Sullivan, et al., 2018). Briefly, 0.1 % CS solutions were prepared by dissolving CS in a 1 % v/v aqueous acetic acid solution and then the pH adjusted to 4.6. CS NPs were prepared by dissolving L. MW CS into 1 % v/v aqueous acetic acid solution whereupon TPP was added in a 3:1 CS:TPP ratio and subsequently the pH of the solution was adjusted to 4.6 using 1 M NaOH. For the nano-solubilisate, the 6 % CASB was heated to 40°C prior to diluting with sterile distilled water to a final concentration of 0.8% of the active component (carnosolic acid) as this was the highest aerosolisable concentration.

3.3.2.2 Preparation, Aerosolisation and Vacuum Skin Packaging of Hake Fillets

Using a sterile knife, hake fillets were cut into ca. 150 g portions and then four pieces of the portioned fish were individually placed on a sterilized custom designed grated shelf that was placed inside a specially modified 7 L container (Sistema, New Zealand) to hold the fish fillets and allow the permeation of the aerosolised antimicrobials on the surface of the hake fillets (Figure 3.1). The lid of the container was tightly closed and 10 mL of 0.1 % w/v CS, 0.1 % w/v CS NP or 0.8 % CASB antimicrobial solutions were loaded into the receiver cup of the atomiser nebuliser (3A, Lonato del Garda, Italy) and aerosolised for 30 min at a rate of 0.35 mL min^{-1} . The aerosolised hake fillets were then removed aseptically from the chamber, and individually placed onto a recyclable polyethylene terephthalate (rPET/PE) tray (590 mm \times 390 mm \times 475 mm; ES Plastic GmbH & Co KG, Germany) and then vacuum skin packed with a

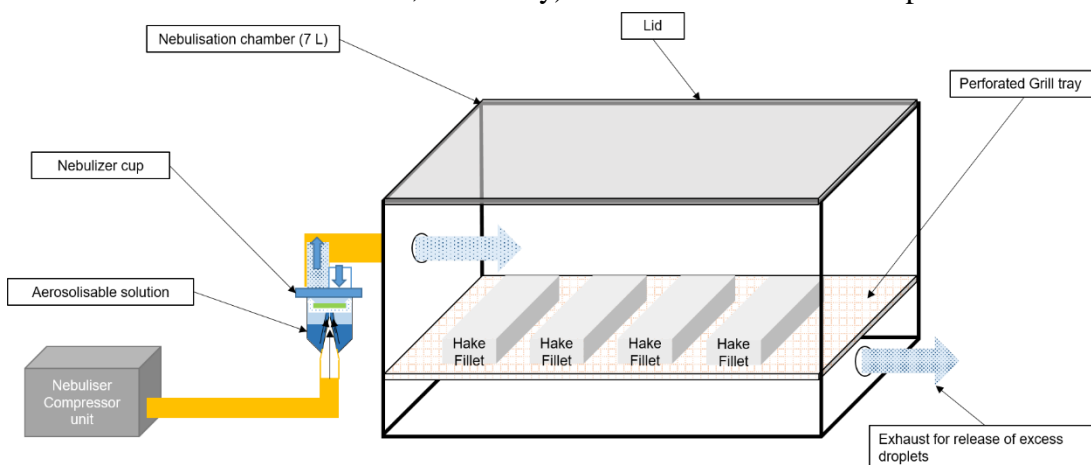


Figure 3.1 Schematic set up of the aerosolisation apparatus

coextruded SkinFreshTop 80 PE/EVOH/PE film (oxygen permeability of $< 5 \text{ cm}^3 \text{ m}^{-2} \text{ dbar}$) using an ILPRA FP Basic VG machine (Vigevano, Italy). VSP hake fillets were stored at 4°C until use and sampling was carried out every three days and for each sampling day, two trays were randomly selected for microbiological and physicochemical analysis. The whole experiment was repeated independently three times and results are the average of 6 replicates.

3.3.3 Physiochemical Analysis of Hake Fillets

3.3.3.1 Proximal Analysis

Fat and moisture of hake fillets were determined using the CEM Analysis System (CEM Corporation, Matthews, NC 28105, USA) (Bostian, Fish, Webb, & Arey, 1985); protein content was determined according to AOAC Procedures (1999) (method 981.10). The ash content of the hake fillets were determined by incineration of the hake samples in a furnace (Nabertherm, Model L9/C6, Nabertherm, Germany) at 550 °C.

3.3.3.2 pH

The pH of the hake fillets were measured using a previously calibrated digital pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland) by inserting the glass probe directly into the hake flesh. Each value represents the average of 7 measurements.

3.3.3.3 Colour

The surface colour of hake fillets during chilled storage at 4 °C was recorded using a Minolta chromameter (CR-300, Minolta Camera Co., Osaka, Japan) as outlined by (Azlin-Hasim, Cruz-Romero, Morris, Cummins, & Kerry, 2015). Briefly, 10 random areas of the hake surface were measured per sample on each measurement day and the average values of ten readings of CIE *L*-value (lightness), *a*-value (redness) and *b*-value (yellowness) were recorded. Whitening index (WI) was calculated using Eq. (3.1) (Borchert, et al., 2014):

$$WI = L - (3b) + (3a) \quad (3.1)$$

3.3.3.4 Lipid Oxidation (TBARS)

The lipid oxidation of hake fillets were assessed by measuring the 2-thiobarbituric acid-reactive substances (TBARS) assay as outlined by (Siu & Draper, 1978) and the malondialdehyde (MDA) content was calculated using an extinction coefficient of $1.56 \times 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$ and results were expressed in mg MDA kg^{-1} of hake fillets.

3.3.4 Microbiological Analysis

In order to obtain a representative sample, 10 g of hake flesh was aseptically taken (5 g of hake flesh from the top side and 5 g from bottom side of the hake fillet) and placed in a sterile stomacher filter bag. Then, 90 ml of sterile MRD was added aseptically into the stomacher bag and homogenised for 180 s using BA6021 stomacher 400 (Colworth, Bury St. Edmunds, UK). The homogenate was then ten-fold diluted and used for the enumeration of total viable counts (TVC), psychrotrophic bacteria, *Pseudomonas* spp., lactic acid bacteria (LAB), total coliforms and *Escherichia coli* (*E. coli*), anaerobic bacteria, and H_2S -producing bacteria. TVC and psychotropic bacteria analysis was determined on PCA using the pour plate method and incubated at 37 °C for 48 h or 4 °C for 1 week, respectively. Lactic acid bacteria (LAB) was enumerated on de Man, Rogosa and Sharpe (MRS) agar using a pour plate method with an overlay and incubated at 30 °C for 72 h. Anaerobic bacteria was enumerated using spread plate method on tryptic soy agar (TSA) containing 0.6 % yeast extract and incubated under anaerobic conditions in anaerobe jars with Anerocult A (Merck) for 72 h at 30 °C. H_2S -producing bacteria was enumerated on Lyngby iron agar after incubation at 30 °C for 72 h. *Pseudomonas* spp. was enumerated using spread plate method after 2 days incubation at 30 °C on *Pseudomonas* agar base to which CFC 0103 supplement was added. For the enumeration of total coliforms and *E. coli*, 1 ml of the appropriate dilution was placed in duplicate onto the centre of Compact Dry-EC chromogenic

plates and incubated at 37 °C for 24 h. As indicated by the manufacturer, blue colonies were counted as *E. coli* and total coliforms included red and blue colonies.

3.3.5 Statistical Analysis

All data was analysed for means and standard deviations, and analysis of variance. One-way analysis of variance of data was carried out using the SPSS 24 for Windows (SPSS Statistical software, IBM Corp., Armonk, NY, USA) software package. Differences between pairs of means was resolved by means of confidence intervals using Tukey's test; the level of significance was set at $P < 0.05$.

3.4 Results and Discussion

3.4.1 Proximal Composition

The proximal composition of hake fillets caught in the Irish Sea are shown in Table 3.1. Similar results were reported by Roncarati, et al., (2012) who found that hake caught in the south Tyrrhenian sea had 79.9, 17.9, 1.2 and 1.54 % moisture, protein, lipid and ash content, respectively. However, Dominguez-Petit, Saborido-Rey, & Medina, (2010) reported that variations in the composition of hake fillets can occur due to environmental and physiological factors such as the nutrition, catching season (spawning cycles), sexual variation, fish size, living area, as well as the other environmental conditions. In addition, the variation in composition of hake fillets may affect sensory attributes including: taste, odour, texture, colour, and surface appearance, which control the acceptability of fish as food to the consumer (Ojagh, Rezaei, Razavi, & Hosseini, 2010). Nevertheless, the results from this study indicated that the proximal composition of hake fillets were not affected significantly ($P > 0.05$) by the antimicrobial treatments. This may perhaps be due to the fact that only a thin layer of antimicrobial substance was deposited on the surface of the hake fillets which had a negligible effect on the proximal composition and, to the best of our knowledge,

this has not been reported elsewhere as typically coatings can affect significantly the proximate composition of fish (Yıldız, 2017).

Table 3.1 Proximate composition of control, CS, CS NP, and CASB treated hake fillets analysed on day 0.

Treatment	Protein (%)	Ash (%)	Fat (%)	Moisture (%)
<i>Control</i>	17.57 ± 0.6 ^a	1.07 ± 0.1 ^a	0.9 ± 0.3 ^a	79.73 ± 0.5 ^a
<i>CS</i>	16.82 ± 1.1 ^a	1.19 ± 0.2 ^a	0.5 ± 0.1 ^a	80.59 ± 0.2 ^a
<i>CS NP</i>	17.19 ± 1.0 ^a	1.10 ± 0.1 ^a	0.96 ± 0.8 ^a	79.86 ± 1.5 ^a
<i>CASB</i>	19.95 ± 1.4 ^a	1.10 ± 0.4 ^a	1.25 ± 0.8 ^a	79.88 ± 1.2 ^a

^{a,b} Mean values in the same column with different superscripts are significantly different ($P < 0.05$).

3.4.2 Changes on pH of Hake Fillets during Storage

The pH of untreated control and antimicrobial treated VSP hake fillets stored at 4 °C are shown in Figure 3.2. Independent of the type of treatment used, the pH values of the hake fillets increased significantly ($P < 0.05$) throughout storage until day 6. Moreover, CS and CS NP treated hake fillets were found to increase the pH of hake fillets significantly ($P < 0.05$) compared to untreated control and CASB treatments. Reportedly, the increase in pH of hake fillets over time is due to the accumulation of undesirable alkaline compounds, such as ammonia and trimethylamine (TMA) which arise from microbial action (Garcia-Soto, et al., 2013). Nevertheless, the pH of the hake fillets herein was between 6 and 7 which is the typical pH of fresh fish; however, this will depend on the species and season (Rodrigues, et al., 2016). Moreover, it has been reported that an increase of pH above 7 is indicative of the dissociation of carbonic acid and an accumulation of undesirable alkaline compounds such as such as

ammonia and trimethylamine (TMA), therefore, it is suggested that consumption of hake above pH 7 should be avoided due to the associated negative organoleptic properties (Garcia-Soto, et al., 2013; Volpe, et al., 2015). However, results indicated that the hake fillets remained below pH 7 and these results are in agreement with Garcia-Soto, et al., (2013) who observed no definite pH trend during storage time with respect to antimicrobial treatments. Conversely, Ozyurt, et al., (2012) reported that the incorporation of rosemary extracts on sardines (*Sardionella aurita*) significantly reduced the pH of the fish muscle during chilled storage at 4 °C. Furthermore, it was

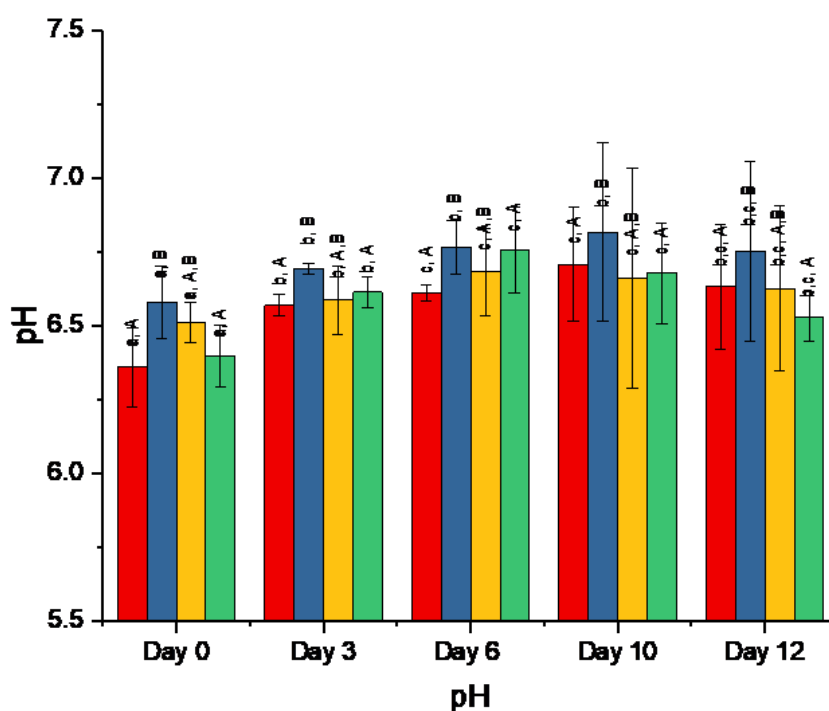


Figure 3.2 Changes in pH of control (■), CS (■), CS NP (■), and CASB (■) treated hake fillets stored at 4°C under vacuum skin packaging over the 12 day storage period. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P<0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P<0.05$).

reported that variations in the initial pH values on fish may be affected by the species of fish, season, and diet, level of activity or stress during the catch as well as type of muscle (Ocano-Higuera, et al., 2011).

3.4.3 Colour of Hake Fillets during Storage

The effect of control, CS, CS NPs or CASB treatments on the CIE *L*-value (lightness), *a*-value (redness), *b*-value (yellowness) and whiteness index (WI) of hake fillets can be seen in Figure 3.3. During storage, independent of the treatment used, lightness (*L*-values) were found to increase significantly ($P<0.05$) until day 9, after which the lightness began to decrease. In addition, CS, CS NP or CASB treated hake fillets were observed to be significantly ($P<0.05$) darker than untreated control hake fillets and a significant ($P<0.05$) difference between CS and CS NP or CASB treated hake fillets was observed. Lower lightness of CS and CS NP treated hake fillets may perhaps be due to the low pH of the solutions used as CS and CS NP were dissolved in 1% acetic acid solution before aerosolisation treatment which may resulted in the leaching of muscle pigments (Mohan, Ravishankar, Lalitha, & Srinivasa Gopal, 2012). In relation to CASB, the dark brown/olive colour of the aerosolisable solution may perhaps be reducing the lightness on the hake fillet surface (Figure 3.3 a.). Regarding redness (*a*-values), independent of the treatment used, the *a*-values decrease significantly ($P<0.05$) after day 9 (Figure 3.3 b). Moreover, CS NPs treated hake fillets had significantly ($P<0.05$) higher *a*-values compared to untreated control, CS or CASB treated hake fillets and may perhaps be due to the strong chelating ability of CS NPs coordinating to pro-oxidant materials decreasing lipid oxidation (Yen, Yang, & Mau, 2008) while also increasing the stability of any red pigments present (Sickler, Claus, Marriott, Eigel, & Wang, 2013). Furthermore, it has been reported that the TPP component of CS NPs can improve redness, potentially due to it delaying the

hydrolysis of meat components (Ghaderi-Ghahfarokhi, Barzegar, Sahari, & Azizi, 2016). Overall, hake fillets have relatively low redness values due to naturally low concentration of “redness” increasing pigments such as carotenoids and/or hemopigments (Sanchez-Zapata, Perez-Alvarez, Fernandez-Lopez, & Barber, 2010). With respect to yellowness (*b*-values), a significant ($P<0.05$) increase was observed up to day 9, independent of the treatment used (Figure 3.3 c.). With respect to antimicrobial treatments, it was observed that untreated control hake differed

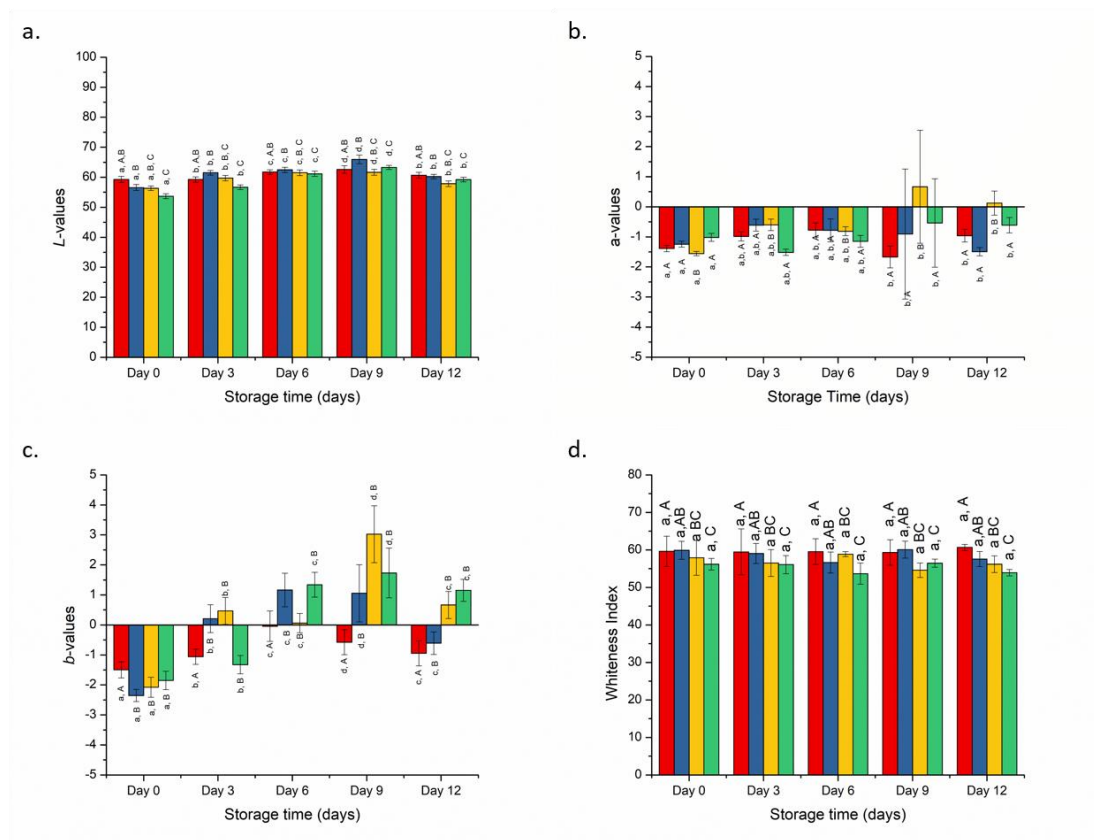


Figure 3.3 Lightness (L-values) (a.), redness (a-values) (b.), yellowness (b-values) (c.), and whiteness index (d.) of control (■), CS (■), CS NP (■), and CASB (■) treated hake fillets stored at 4°C under vacuum skin packaging over the 12 day storage period. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P<0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P<0.05$).

significantly ($P < 0.05$) to CS, CS NP, or CASB treated hake fillets. The increase in b -values may be associated with the increased oxidation and formation of metmyoglobin (Ghaderi-Ghahfarokhi, et al., 2016). Regarding the WI, a significant ($P < 0.05$) difference between control, CS, and CS NP or CASB treated hake fillets was observed (Figure 3.3.d.). Overall, control treated hake fillets had the highest WI, while the lowest WI was obtained when hake fillets were aerolised with CASB.

3.4.4 Lipid Oxidation (TBARS) of Hake Fillets during Storage

The lipid oxidation of control, CS, CS NP, and CASB treated hake fillets are shown in Figure 3.4. It was observed that, independent of the antimicrobial treatment used, the TBARS values decreased significantly ($P < 0.05$). However, while untreated control hake fillets were found to have a higher TBARS than CS, CS NP or CASB treated hake fillets, this was not statistically significant. It was reported that the decrease in TBARS may be as a result of the reaction between malondialdehyde and components of the fish muscle such as proteins, amino acids and glycogen which will decrease the amount of malondialdehyde present (Shahbazi, et al., 2018). Furthermore, the antimicrobials used herein have all been reported to have antioxidant properties which may perhaps be “mopping” up lipid oxidation initiators and; therefore, reduce lipid oxidation pathways (Ojagh, et al., 2010; Shahbazi, et al., 2018; Yang, et al., 2016). It has been reported that the antioxidant mechanism of action of CS is related to the metal-chelation ability of CS to form a complex with lipids and good scavenging ability on hydroxyl radicals and that the antioxidant properties are increased with the degree of N-deacetylation (Kim & Thomas, 2007). Conversely, CASB which is derived from rosemary essential oils, has a phenolic structure which have been shown to possess remarkable antioxidant activity due to their ability to capture chain-carrying lipid peroxy radicals associated with lipid oxidation and quench their propagation

through a radical-radical reaction (Amorati, Foti, & Valgimigli, 2013). In addition to the antimicrobial treatments used, VSP has been shown to reduce the effect of lipid oxidation, especially in comparison to MAP, due to the presence of CO₂ in MAP denaturing muscle proteins that liberates iron and can acts as pro-oxidant in the lipid fraction; however, in VSP there is negligible CO₂ to initiate lipid oxidation via this process (Perez-Alonso, Aubourg, Rodriguez, & Barros-Velazquez, 2004; Rodrigues, et al., 2016). Nonetheless, independent of the treatment used and the storage period,

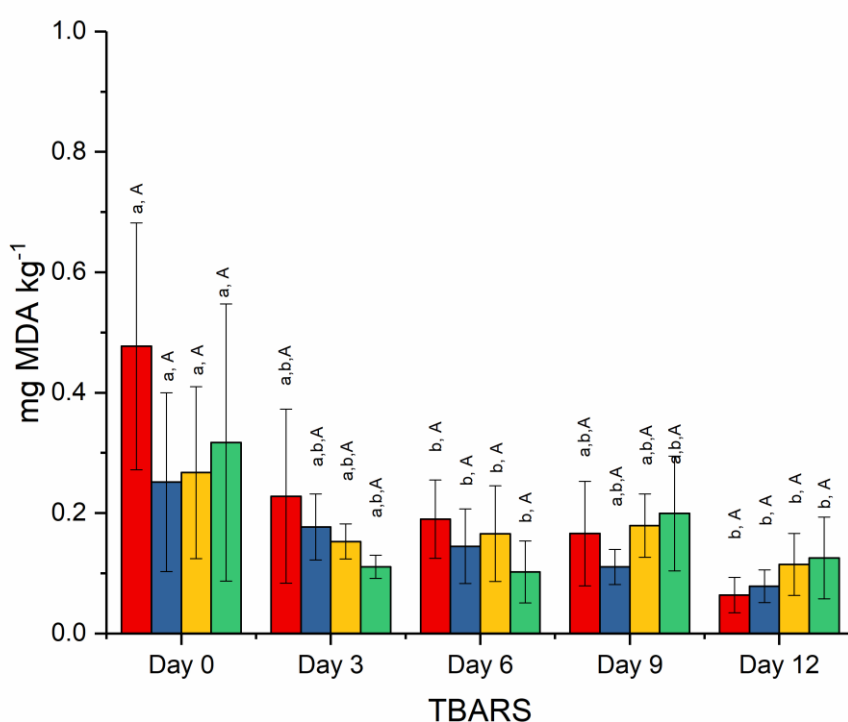


Figure 3.4 Changes in TBAR'S (mg MDA kg⁻¹) of control (■), CS (■), CS NP (■), and CASB (■) treated hake fillets stored at 4°C under vacuum skin packaging over the 12 day storage period. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P < 0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P < 0.05$).

the TBARS remained below the 1.5 mg MDA kg⁻¹ limit beyond which fish will normally develop objectionable odours/tastes (M. Cruz-Romero, Kelly, & Kerry, 2008); however, other authors such as Ojagh, et al., (2010) have suggested that this limit may even be as high as 5 mg MDA/kg for fresh fish. The results reported in this study are in agreement with the results reported by Garcia-Soto, et al., (2013) who found that lipid oxidation is not an important deterioration parameter during chilled storage of lean white fish species such as hake fillets.

3.4.5 Microbiological Analysis of Hake Fillets

From a microbiological perspective, the upper acceptable limit for the TVC of fish products is 7 log CFU g⁻¹ (Carrion-Granda, et al., 2018). However, in this study, a TVC value of 6 log CFU g⁻¹ was set as the maximum limit of acceptability. The effect of antimicrobial treatments in combination with VSP stored at 4°C on the microbial counts of TVC, anaerobes, psychrotrophs, *Pseudomonas* spp., H₂S-producing bacteria, LAB, *E. coli*, and coliforms are shown in Figure 3.5. Over the storage period, a significantly ($P<0.05$) lower bacterial population of TVC, anaerobic and coliform bacteria was observed in hake fillets that were aerolised with CS, CS NP or CASB compared to untreated control hake fillets. While higher microbial populations of LAB, *Pseudomonas* spp., psychrotrophs, *E. coli*, and H₂S-producing bacteria were observed in untreated control treatments compared to CS, CS NP or CASB treated hake fillets; however, these differences were not found to be statistically significant ($P>0.05$).

Regarding TVC, the initial bacterial count for control hake fillets was 4.40 log CFU g⁻¹, while the TVC for hake fillets immediately after treatment with CS, CS NP or CASB, were 4.15, 4.15 and 4.04 log CFU g⁻¹, respectively (Figure 3.5 a.). However, the initial microbial count found in this study was relatively high compared to the

initial TVC of untreated hake fillets caught off the Galician Atlantic coast (North-Western Spain) which was $2.46 \log \text{CFU g}^{-1}$ (Garcia-Soto, et al., 2013). The higher initial TVC of hake fillets observed herein may perhaps be due to poor handling practises post-mortem such as: manual processing, handling, storage conditions, the skinning process, the time taken to reach the fishery plant, and the hygiene condition of the fishery/processing plant during harvesting (Carrion-Granda, et al., 2018; Günlü, et al., 2013). Untreated control hake fillets reached the set limit of acceptability on day 6; however, hake fillets treated with CS, CS NP or CASB reached the acceptability limit after 8, 8.5 and 9 days of storage, respectively. This represents a significant ($P<0.05$) extension of the shelf-life of CS, CS NP, and CASB treated hake fillets upon untreated control fillets of 41, 50 and 55 %, respectively. The longest shelf life was obtained on hake samples treated with CASB; however, this shelf life extension was not significantly different compared to hake fillets treated with CS or CS NPs. While it has been widely reported that CS and CS NPs are more effective antimicrobials materials than essential oil based antimicrobials (L. Wang, et al., 2011), the increased shelf life observed in hake fillets treated with CASB may be due to the higher concentration of CASB used compared to fillets treated with CS or CS NPs in order to exhibit a similar antimicrobial response. However, possible application of a higher concentration of CS or CS NPs solutions for aerosolisation may be required to be studied to increase antimicrobial activity; however, concentration increases of CASB are limited as higher concentrations than 0.8 % are difficult to aerosolise due to an increased viscosity of the solution.

With respect to anaerobic microbial population, a statistical ($P<0.05$) difference between untreated control and CS, CS NP or CASB treated hake fillets was observed (Figure 3.5 b.). The initial anaerobic bacterial count was 6.20, 5.49, 5.43, and 5.46 log

CFU g⁻¹ for untreated control, CS, CS NP, and CASB treated hake fillets, respectively. The initial microbial count of anaerobic bacteria observed herein was high, as other authors such as, Perez-Alonso, et al., (2004), who packaged and stored Atlantic pomfret (*Brama brama*) using VSP at 4 °C found that the initial anaerobe colonies was 4.11 log CFU g⁻¹. The high initial loadings may perhaps be due afore mentioned handling conditions. Nonetheless, a significant ($P<0.05$) reduction in the anaerobic population of CS, CS NP or CASB treated hake fillets were observed compare to untreated control hake fillet samples.

Regarding psychrotrophic bacteria, results indicate that untreated control hake fillets had greater spoilage compared to CS, CS NP and CASB treated hake fillets; however, this difference was not statistically significant ($P>0.05$) (Figure 3.5 c.). In addition, it was reported that the rate of growth of psychrotrophic bacteria over the storage period was higher compared to TVC and may perhaps be due to the chilled storage of hake fillets at 4 °C preferentially encouraging the growth of psychrotrophic bacteria over mesophilic bacteria (TVC) (Azlin-Hasim, et al., 2015).

Similarly, results from *Pseudomonas* spp. bacteria, indicate that untreated control hake fillets had higher microbial spoilage compared to CS, CS NP and CASB treated hake fillets; however, this was not a statistically significant ($P>0.05$) difference (Figure 3.5 d.). From a microbiological perspective the upper limit of 7 log CFU g⁻¹ for *Pseudomonas* spp. (Shahbazi, et al., 2018) bacteria was not surpassed until day 6 for untreated control hake fillets and day 12 for CS treatments while neither CS NP or CASB treated hake fillets surpassed the upper limit perhaps due to the antimicrobial effect of the treatments.

Regarding H₂S-producing bacteria, results indicate that untreated control hake fillets had higher microbial spoilage compared to CS, CS NP and CASB treated hake fillets; however, this difference was not statistically significant ($P>0.05$) (Figure 3.5 e.) From a microbiological perspective, the upper acceptable limit for the H₂S producing bacteria of fish products is 6 log CFU g⁻¹ (Carrion-Granda, et al., 2018) and the upper level of microbial acceptability was surpassed after day 2 of storage for untreated control hake fillets and on day 3 for CS, CS NP or CASB treated hake fillets (Carrion-Granda, et al., 2018). These results are in contrast to (Carrion-Granda, et al., 2018), who used whey protein isolate coatings incorporated with EOs combined with MAP on hake fillets and found that H₂S-producing bacteria were negligible on day 0. In addition, it has been reported that once *S. putrefaciens* reaches its upper acceptability limit, the bacteria starts producing sulphur compounds resulting in the spoilage of fish products and an increase in negative organoleptic properties. The high initial microbial count of H₂S-producing bacteria may perhaps be due to afore mentioned handling condition and H₂S-producing bacteria favouring low oxygen environments such as MAP and VSP due to their ability to anaerobically respire (Fernandez-Saiz, et al., 2013).

With respect to LAB bacteria, no significant ($P>0.05$) difference between treatments was observed; however, the microbial load remained below the upper acceptability limit of 9 log CFU g⁻¹ (O'Neill, Cruz-Romero, Duffy, & Kerry, 2018) throughout the observed storage period (Figure 3.5 f.). However, it has been reported that LAB are not a major spoilage microorganism for fish due to the low storage temperature and the development of an antagonist microflora (Galli, Franzetti, Carelli, Piergiovanni, & Fava, 1993) and our results are in agreement with this. Moreover, the results herein, indicate that Gram-negative bacteria were apparently more susceptible than Gram-

positive bacteria, and these results are in agreement with Azlin-Hasim, et al., (2015) and Galli, et al., (1993). Conversely, Fernandez-Saiz, et al., (2013) reported that Gram-positive LAB was the more dominant species than Gram-negative bacteria on hake fillets wrapped in a combination of CS films. The reduced effect of CS, CS NP or CASB treated hake fillets with respect to LAB bacteria, may perhaps be due to their greater resistance to hurdles such as high carbon dioxide levels (found in MAP) (Carrion-Granda, et al., 2018) and vacuum-packaging (Fernandez-Saiz, et al., 2013). Furthermore, Gram-positive bacteria possessing a thicker peptidoglycan layer than Gram-negative, therefore, making it difficult for antimicrobial materials to penetrate into the cells wall (Azlin-Hasim, et al., 2015).

Regarding *E. coli*, no significant ($P>0.05$) difference between treatments was observed; however, the microbial load of *E. coli* bacteria remained under the acceptability limit of $2.69 \log \text{CFU g}^{-1}$ throughout the observed storage period (O'Neill, et al., 2018). Initially counts for *E. coli* bacteria were low to non-existent and the low initial loadings are in agreement with Fernandez-Saiz, et al., (2013) (Figure 3.5 g.). However, over the storage period microbial counts of *E. coli* bacteria increased significantly ($P<0.05$) until day 6 after which they remained relatively constant. While *E. coli* is a facultative anaerobe, it did not surpass the upper acceptability limit during this period; however, *E. coli* is a serious food spoilage microorganism and its excessive presence in fish can also be indicative of contamination from sources such as sewage in its local environment (Fernandez-Saiz, et al., 2013).

Regarding coliforms, a significant ($P<0.05$) difference between untreated control fillets and CS, CS NP and CASB treated fillets was observed (Figure 3.5 h.). Furthermore, independent of treatment, a significant increase in the microbial population of coliform bacteria was observed; however, the microbial load did not

surpass the upper acceptability limits of 5 log CFU g⁻¹ (García-Soto, Fernández-No, Barros-Velázquez, & Aubourg, 2014). Low initial counts of coliform bacteria have been reported elsewhere by Volpe, et al. (2015) who coated rainbow trout fillets with a carrageenan enriched with essential lemon oil. These results indicate good hygiene conditions in the fishery plant (Shahbazi, et al., 2018). When comparing the shelf life of CS, CS NP, and CASB treated hake fillets, nanomaterials treatments of CS NPs and CASB gave a marginally longer shelf-life than non-nano CS; however, this was not statistically significant ($P>0.05$). This may be due to the fact that the antimicrobial activity of CS and CS NP materials have been reported to be similar due to the strong solvent swelling character and CS:TPP composition of CS NPs affecting the amount of free amine and therefore, the antimicrobial activity of CS NPs (Ristić, Lasić, Kosalec, Bračić, & Fras-Zemljič, 2015; D. J. Sullivan, et al., 2018). Moreover, CS and CS NP have a similar mode of antimicrobial action which is not fully understood; however, several modes of action have been proposed such as: (i) positively charged CS interacting with the negatively charged components of the bacterial cell walls, resulting in cytoplasm leakage from the microorganism, (ii) through its ability to form a layer surrounding the bacterial cell, therefore, inhibiting the absorption of nutrients, and resulting in cell death (David Joseph Sullivan, et al., 2018), or (iii) low molecular weight CS can bind to DNA and inhibit RNA replication due to its ability to penetrate towards the nuclei of microorganisms. Furthermore, the Gram-strain may also affect the mode of antimicrobial action as high molecular weight CS can form a film on Gram-positive bacteria preventing uptake of nutrients while low molecular weight CS could interfere with cellular metabolism on Gram-negative bacteria due to penetration of CS into the cell (D. J. Sullivan, et al., 2018).

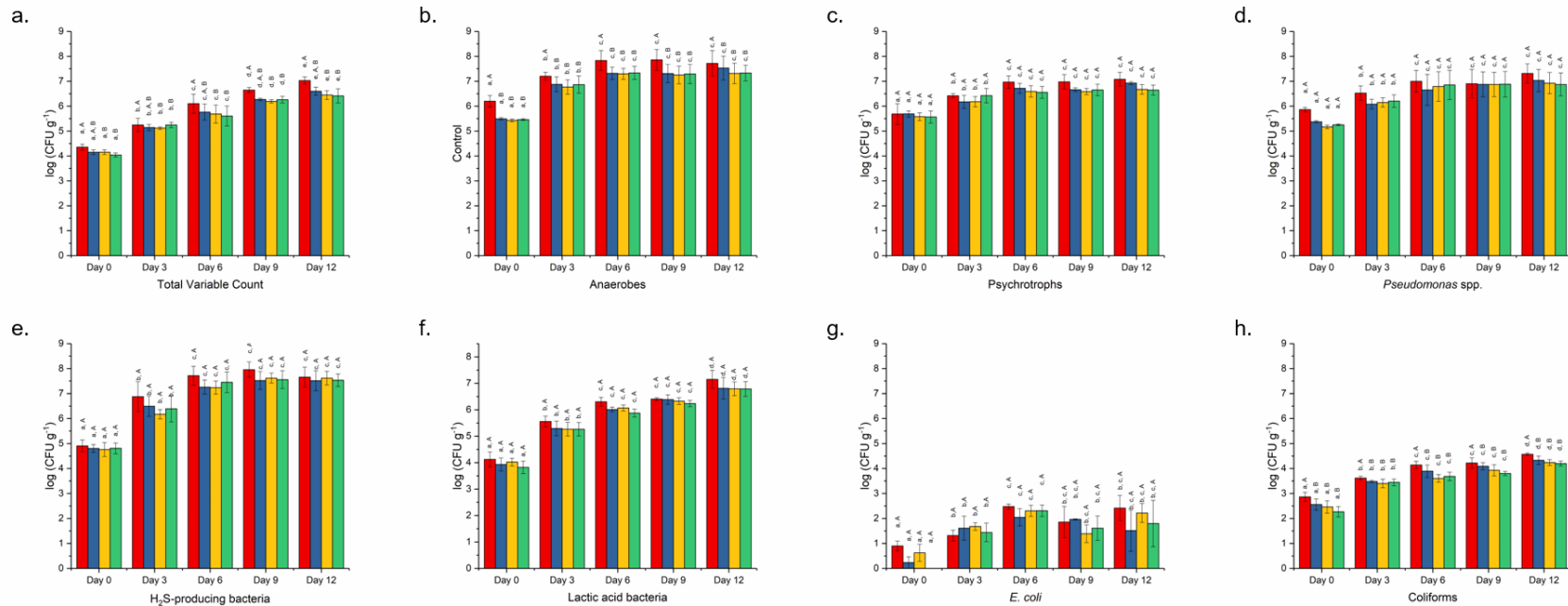


Figure 3.5 Microbiological changes of (a) TVC, (b.) anaerobic bacteria (c) psychrotrophic bacteria, (d) *Pseudomonas* spp., (e.) H₂S-producing bacteria, (f.) LAB, (g.) *E. coli*, and (h.) total coliform for control (■), CS (■), CS NP (■), and CASB (■) treated hake fillets stored at 4°C under vacuum skin packaging over the 12 day storage period. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P < 0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P < 0.05$).

Regarding the marginally longer shelf life seen by CS NPs, may perhaps be associated with NPs increased surface area compared to their bulk counterpart thus allowing more interaction with the bacterial cell components (Ngan, et al., 2014) and in addition CS NPs greater monodispersity may allow for more efficient aerosolisation and therefore, a greater antimicrobial coating on the hake fillet surface. Regarding CASB, the mode of antimicrobial activity differs from CS and CS NP and likewise, the exact antimicrobial mechanism of action of EO's based materials is not fully understood; however, it is believed to be from the synergistic action of constituent secondary metabolites commonly found in EO's of clove, oregano, rosemary, thyme or sage. Carsonic acid is phenol diterpenoid that has been associated with the antimicrobial activity of rosemary essential oil (Abdollahi, et al., 2014) and is a compound that can affect the cellular membrane allowing for the passive transport of ions through the membrane and can disintegrate outer membranes of Gram-negative bacteria or in Gram-positive bacteria alter the membrane permeability, allowing permeation cations like H^+ and K^+ (David Joseph Sullivan, et al., 2018). Moreover their greater antimicrobial activity may perhaps be due to enhanced antimicrobial activity of nano-solubilisates (M. C. Cruz-Romero, Murphy, Morris, Cummins, & Kerry, 2013) and afore mentioned concentration difference.

Overall, the results confirmed that the application of natural antimicrobials can enhance the shelf life of fish products but that the initial microbial load will affect significantly the shelf life of these products; therefore, to enhance safety and shelf life of fish products through the application of any natural antimicrobial should start with the highest quality product as shelf life enhancement will be defined by the initial bacterial count and as for any preservation technology, the initial quality of fish products cannot be enhanced.

3.5 Conclusions

Results obtained herein indicate that the microbiological and physiochemical properties of hake fillets aerosolised with CS, CS NP or CASB antimicrobial coating solutions as part of a hurdle strategy significantly ($P < 0.05$) enhanced shelf life in comparison to control treated hake fillets. The best shelf life was observed using CASB treatments which increased the shelf life by up to 50 % upon control treatments. The physiochemical properties were also improved after the application of aerosolised antimicrobial materials and without a significant effect on the proximate composition of hake fillets. Therefore, aerosolisation treatment of hake fillets with NAM antimicrobial solutions was an effective hurdle in VSP packed hake fillets. This research highlights the potential of aerosolisation treatments which require small volumes of antimicrobial solutions, give good coating coverage, and have a minimal impact on physiochemical parameters such as proximal analysis. Moreover aerosolisation treatment could be a suitable method to treat a diverse range of high value or perishable food products including: fruits, vegetables, chicken, beef and seafood. Additionally, the shelf extension from aerosolisation treatments could be further improved upon through the manipulation of aerosolisation parameters such as flow rate, antimicrobial solution concentration and exposure times and use of other antimicrobial nanomaterial solutions such as EO nanoemulsions and these require further studies. Overall the results of this study demonstrate that the aerosolisation treatments of nano and non-nano natural antimicrobial materials are an effective hurdle to extend the shelf-life of hake fillets.

3.6 References

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Chapter 4: Development of Monodisperse Oregano Nanoemulsions for Aerosolisation Applications as part of a Hurdle Strategy to Extend the Shelf-life of Hake Fillets (*Merluccius Merluccius*)

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This Chapter is in preparation for submission to an academic journal.

4.1 Abstract

The objectives of this study were to evaluate the effects of different surfactants (Tween® 20 and Pluronic™ 127), the surfactant to oil ratio (2:1, 1:1, and 1:2) and physical treatments (untreated, IKA ultra-turrax or tip-sonication) on the droplet size, polydispersity index and zeta potential of oregano nanoemulsions. Antimicrobial activity of the monodisperse nanoemulsions was then assessed against cultures of *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas fluorescens*. The nanoemulsion with the greatest properties was tip-sonicated, Tween® 20 surfactant nanoemulsions possessing a surfactant to oil ratio of 1:1, an average droplet diameter of 131 nm and a zeta potential of 39.2 mV showed the greatest antimicrobial activity. Therefore, these nanoemulsions were applied via aerosolisation treatment on fresh European Hake (*Merluccius merluccius*) fillets caught in Irish waters. The application of aerosolisation treatment to hake fillets extended ($P<0.05$) product shelf-life by up to 50% when compared to untreated control hake fillets. Findings from this study demonstrated that monodisperse oregano nanoemulsions can be obtained using a variety of treatment techniques and that their application to foods, and possibly packaging materials, could be used to enhance the shelf-life of food products.

4.2 Introduction

Numerous natural antimicrobial materials (NAMs) have been identified for potential application in the food industry, and of particular interest, are the essential oils (EO's) which have been shown to exhibit strong antimicrobial and antioxidant activities in food systems. According to the ISO (2013), EO's are products obtained by steam distillation from a natural raw material of plant origin. Moreover, EOs possess a favourable ingredient

status, such as; GRAS (Generally Recognised as Safe) by the Food and Drug Administration (FDA) and are generally acceptable to consumer owing to their historical use as natural flavourings and medicines, thereby making them suitable candidates for use as safety and spoilage hurdles in food applications (Burt, 2004; Holley & Patel, 2005; Hyldgaard, Mygind, & Meyer, 2012; Sullivan, Azlin-Hasim, et al., 2018; Turek & Stintzing, 2013). Compositionally, EO's are made up of a variety of secondary metabolite constituents such as carvacol, thymol, p-cymene among others. These metabolites synergistically contribute to the antimicrobial activity of EO's via interactions with bacterial cell walls and to the antioxidant activity, through capturing chain-carrying lipid peroxy radicals associated with lipid oxidation (Amorati, Foti, & Valgimigli, 2013; Hyldgaard, et al., 2012).

Nonetheless, roadblocks in EO applications exist, such as; their hydrophobic character, immiscibility with aqueous phases, and a strong impact on organoleptic properties which limit their use in food products. However, strategies have been developed to overcome these limitations, such as the development of nano-emulsification strategies of EO's which has been shown to improve properties, such as; stability (Moraes-Lovison, et al., 2017), (Alejandra Acevedo-Fani, Soliva-Fortuny, & Martín-Belloso, 2017), antimicrobial activity (Noori, Zeynali, & Almasi, 2018) and reduce the negative impact on organoleptic properties through controlled release of antimicrobial volatile constituents (Paparella, et al., 2016). Nanoemulsions can be developed via the application of high-energy methods that utilise mechanical or ultrasound devices to generate shearing (rotor-stator) or pressure difference (sonication), to decompose the emulsion structures into nanoemulsions (Spinelli, Mansur, González, & Lucas, 2010). Nanoemulsion droplet size is also affected

by physicochemical properties of the oil and emulsifier, their relative concentrations to each other, the interfacial tension and viscosity and these factors will also influence the amount of energy required to form nanoemulsions (Donsì & Ferrari, 2016; Komaiko & McClements, 2016). Regarding their stability, nanoemulsions are more stable than microemulsions as they are less susceptible to the effects of gravitational forces and sedimentation due to greater Brownian motion and diffusion rate, however, they are more susceptible to Ostwald ripening (McClements, 2011; Tadros, Izquierdo, Esquena, & Solans, 2004). Nevertheless, the application of EO nanoemulsions on food products has steadily increased in recent years and they have been applied or incorporated on various food products, such as: lettuce via dip-coating (Bhargava, Conti, da Rocha, & Zhang, 2015), green beans using spray-coating (Severino, et al., 2015), milk (Hilbig, Ma, Davidson, Weiss, & Zhong, 2016), chicken pate (Moraes-Lovison, et al., 2017) and on chicken breast as an edible coating (Noori, et al., 2018).

Additionally, the unique physicochemical properties of nanoemulsions, such as their nano-scale droplet size make them ideal candidate materials for application *via* aerosolisation. Aerosolisation is defined as the dispersion of a liquid material into air, in the form of fine mist, usually for sanitary purposes, especially for respiratory medical treatments and room disinfection (Andersen, et al., 2006). This type of delivery offers a number of advantages, including relative ease of application and higher surface coverage owing to the small droplet size compared to conventional spray-coating size (Jiang, et al., 2017). Currently, this treatment method has been limited to organic acid sanitisers and disinfectants, such as peroxyacetic acid and sodium hypochlorite, due to their water

solubility and broad antimicrobial activity and have been applied to various fruits such as cherry tomatoes (Jiang, et al., 2017) and strawberries (Vardar, Ilhan, & Karabulut, 2012).

Hake fillets (*Merluccius merluccius*) are a widely consumed fish product, predominantly in European markets. However, hake fillets, like all seafood products, are highly perishable and deteriorate rapidly post-mortem due to various biochemical and microbial mechanisms as a result of possessing high water content, mild pH and the presence of large quantities of non-protein nitrogen, among other factors, thereby contributing to a relatively short shelf-life (Fernandez-Saiz, Sanchez, Soler, Lagaron, & Ocio, 2013; Garcia-Soto, Aubourg, Calo-Mata, & Barros-Velazquez, 2013; Otero, Perez-Mateos, & Lopez-Caballero, 2017). Additionally, it has been estimated that up to 30–50% of such product is lost through spoilage throughout the supply chain (Garcia, et al., 2015), making the preservation of harvested fish more critical, particularly in light of growing future demands that will exist globally for valuable protein-based food sources.

Studies have previously reported on nanoemulsion processing parameters such as surfactant-type or surfactant-to-oil (SOR) utilisation, on treatments using nanoemulsions which were untreated, treated by IKA or by employing tip-sonication which were investigated individually, or in combination, affecting the droplet size and monodispersity of the EO nanoemulsions (Akbas, Soyler, & Oztop, 2018; Carrion-Granda, Fernandez-Pan, Rovira, & Mate, 2018; Ma, Davidson, & Zhong, 2016). Additionally, the application of these materials have been reported through various technologies, including: direct incorporation into the polymeric packaging material (Yang, et al., 2016), coating onto packaging material surfaces (Erkan, 2012), and as part of an inherently antimicrobial film-forming polymer (such as CS) (Shahbazi & Shavisi, 2018). However, to the best of our

knowledge, none have reported on the application of monodisperse EO nanoemulsions via aerosolisation treatments to fish products. Therefore, the objectives of this study were to assess the effect of surfactant-type or SOR nanoemulsions, and treatments including untreated, IKA or tip-sonication on the monodispersity of oregano nanoemulsions and subsequently, assess aerosolisation treatment of the most suitable nanoemulsion on hake fillets as part of a hurdle strategy to extend product shelf-life. Synthesised nanomaterials were subsequently characterised and their effect on the microbiological and physiochemical properties of hake fillets assessed.

4.3. Materials & Methods

4.3.1 Materials

Oregano essential oil was purchased from Lionel Hitchens Ltd (Barton Stacey, Hampshire, UK). Tween 20® (T20) and resazurin salt were purchased from Sigma Aldrich (St. Louis, Missouri, USA) while Pluronic™ 127 (P127) was purchased from the BASF Corporation (Florham Park, New Jersey, USA). Cuvettes for zeta potential and particle size analysis were purchased and used as received from Malvern (Malvern Instruments Ltd, Malvern, UK). Mueller-Hinton Broth (MHB) and Maximum Recovery Diluent (MRD) were purchased from Oxoid (Basingstoke, UK). *Escherichia coli* (*E. coli*) (NCIMB 11943), *Bacillus cereus* (*B. cereus*) (NCIMB 9373), *Staphylococcus aureus* (*S. aureus*) (NCIMB 13062) and *Pseudomonas fluorescens* (*P. fluorescens*) (NCIMB 9046) were maintained on Tryptic Soy Agar slants until use at 4°C. Freshly caught skinned Hake fillets (hitherto referred to as hake fillets) were purchased from Ballycotton Seafood Ltd (Garryvoe, Ireland). Media for microbiological analysis including; plate count agar, De Man, Rogosa and Sharpe (MRS) agar and *Pseudomonas* Agar Base with selective

supplement CFC (cetrimide, fucidin, cephaloridine) (SR0103) was purchased from Oxoid, Tryptic Soy Agar was purchased from Merck and Compact Dry-EC chromogenic plates were obtained from Nissui Pharmaceutical (Co. Ltd. Japan). Lyngby agar was made using (g L⁻¹); Peptone (20), bacteriological agar (15), sodium chloride (5), sodium thiosulfate (0.3), yeast extract (3), beef extract (3), L-cysteine (0.6) and ferric citrate (0.3) which were all purchased from Sigma Aldrich.

4.3.2 Development of Oregano Nanoemulsions

Oregano essential oil (OEO) was weighted out at 3.2% w/v based upon four-times the highest (8.8mg mL⁻¹) minimum inhibition concentration (MIC) reported by Rodríguez-Calleja, Cruz-Romero, García-López, & Kerry, (2015). Non-ionic Tween 20® (T20) and polymeric Pluronic 127® (P127) were used as the surfactants due to their different physical properties and were added to the OEO in oil to surfactant ratios (SOR) of 2:1, 1:1, and 1:2. These solutions were then subjected to droplet size reducing physical treatments including; IKA Ultra-Turrax™ which was carried out using a DT-20 Tube with rotor-stator homogeniser at 4600 rpm for 2 min (IKA Werke, Janke & Kunkel GmbH & Co KG, Staufen, Germany) and tip-sonication in an ice bath using a 2mm probe (EpiShear™ Probe Sonicator, Active Motif, UK) at 20kHz frequency with an amplitude of 80% for 10 min in 30s burst with 10s rests.

4.3.3 Characterisation of Oregano Nanoemulsions

Particle size analysis and zeta potential measurements of OEO nanoemulsions were carried out using a Malvern Zetasizer Nano Series HT (Malvern, U.K.). For particle size analysis nanoemulsion solutions were loaded into a disposable cell (ZEN0040) and analysis performed at 25°C using a scattering angle of 173°. Particle size distribution was

determined using the Mark–Houwink and Smoluchowski model. For zeta potential analysis, disposable folded capillary tubes (DTS1070) were filled with nanoemulsion solutions and evaluated in automatic mode. OEO nanoemulsion solutions were analysed in duplicate with 3 measurements per sample. Nanoemulsions were prepared for AFM analysis by diluting the emulsion solution 10-fold in deionised water and were then spin-coated onto clean Si coupons at 3000rpm for 30s (Specialty Coating Systems, 6800 spin coat series, IN, USA) and allowed to air dry for 4h at 25°C. Atomic Force Microscope (SPM, Park systems, XE-100, South Korea) analysis was carried out using enhanced resolution NCHR type tips in non-contact mode under ambient conditions with a force constant of 60 000 kN m⁻¹ and a scanning force of 0.11 nN where topographic and phase images were recorded simultaneously.

4.3.4 Antimicrobial Activity Assay

In this study, the antimicrobial activity of tip-sonicated T20 and P127 nanoemulsions against Gram-positive bacteria (*Staphylococcus aureus* (*S. aureus*) (NCIMB 13062) and *Bacillus cereus* (*B. cereus*) (NCIMB 9373)) and Gram-negative bacteria (*Escherichia coli* (*E. coli*) (NCIMB 11943) and *Pseudomonas fluorescens* (*P. fluorescens*) (NCIMB 9046)) was assessed. Before use, all pure culture bacteria were grown for 18h at 30°C (*P. fluorescens* and *B. cereus*) or 37°C (*S. aureus* and *E. coli*) in Mueller-Hinton broth (MHB) (Oxoid, UK) under constant agitation at 170rpm on an orbital shaker (Innova 2300, New Brunswick™, Germany).

4.3.4.1 Minimum Inhibition Concentration

The antimicrobial activity of T20 and P127 nanoemulsions were measured by determining the minimum inhibitory concentration (MIC) against the target microorganisms in a 96-

flat bottom well tissue culture microplates (Sarstedt Inc., NC, USA) according to the NCCLS (2000) broth microdilution method as described by (M. C. Cruz-Romero, Murphy, Morris, Cummins, & Kerry, 2013). Bacterial strains were cultured overnight at the appropriate temperature and adjusted to a final density of 10^5 CFU mL⁻¹ using maximum recovery diluent, and used as an inoculum within 15 min of preparation. Briefly, 100µL of double-strength MHB (2XMHB) was added to each well in rows A to F, 200µL of adjusted bacterial culture suspension was added to row H in columns 1–11 and 200µL of sterile 2XMHB was added to column 12. In each well of row G, 150µL of 3.2 w/w% OEO nanoemulsions were dispensed and a three-fold serial dilution was performed by transferring 50µL of antimicrobial solutions from row G into the corresponding wells of row F through to row B. After mixing, 50µL of the resultant mixture on row B was discarded. Finally, using a 12-channel electronic pipette (Model EDP3-Plus, Rainin, USA) 15µL of the tested microorganisms was pipetted from each well in row H into the corresponding wells in row A followed by rows B to G. Positive (Row A) and negative growth controls (Column 12) were included in each assay plate. The inoculated plates were incubated in a wet chamber for 24hr at 30°C (*P. fluorescens* and *B. cereus*) or 37°C (*E. coli* and *S. aureus*). Resazurin (0.015%) was added to wells A–F (30µL per well), and further incubated for 2hr for the where columns with no colour change (resazurins blue colour remained unchanged if no microbial growth; however, will turn pink if microbial growth occurs) were scored as above the MIC value. The lowest concentration showing inhibition of growth was considered to be the MIC for the target microorganisms. The assay was repeated in triplicate.

4.3.5 Preparation and Aerosolisation of Antimicrobials onto Hake Fillets

4.3.5.1 Preparation of Oregano Essential Oil Nanoemulsion Solution

From the results of this study, T20-OEO (1:1) was chosen as the antimicrobial solution for nebulisation due to its small droplet size and good antimicrobial activity and was formulated as outlined previously.

4.3.5.2 Preparation, Aerosolisation and Vacuum Skin Packaging of Hake fillets

Using a sterile knife, hake fillets were cut into ca. 150g portions and then four pieces of the portioned fish were individually placed on a sterilized custom designed grated shelf that was placed inside a specially modified 7L container (Sistema, New Zealand) to hold the fish fillets and allow the permeation of the nebulised antimicrobials on the surface of the hake fillets. The lid of the container was tightly closed and 10mL of T20-OEO (1:1) antimicrobial solution was loaded into the receiver cup of the atomiser nebuliser (3A, Lonato del Garda, Italy) and nebulised for 30min at a rate of 0.35 mL min^{-1} . The nebulised hake fillets were then removed aseptically from the chamber, and individually placed onto a recyclable polyethylene terephthalate (rPET/PE) tray (590mm \times 390mm \times 475mm; ES Plastic GmbH & Co KG, Germany) and then vacuum-skin packed with a coextruded SkinFreshTop 80 PE/EVOH/PE film (oxygen permeability of $<5 \text{ cm}^3 \text{ m}^{-2} \text{ day}$ at standard pressure and temperature) using an ILPRA FP Basic VG machine (Vigevano, Italy). VSP hake fillets were stored at 4°C until use and sampling was carried out every three days and for each sampling day, two trays were randomly selected for microbiological and physicochemical analysis. The whole experiment was repeated independently three times and results are the average of 6 replicates.

4.3.5.3 Physiochemical Analysis

4.3.5.3.1 Proximal Analysis

Fat and moisture of hake fillets were determined using the CEM Analysis System (CEM Corporation, Matthews, NC 28105, USA) (Bostian, Fish, Webb, & Arey, 1985); protein content was determined according to AOAC Procedures (1999) (method 981.10). The ash content of the skinned hake fillets were determined by incineration of the hake samples in a furnace (Nabertherm, Model L9/C6, Nabertherm, Germany) at 550°C.

4.3.5.3.2 pH

The pH of the hake fillets were measured using a digital pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland) by inserting the glass probe directly into the hake flesh.

4.3.5.3.3 Colour

The surface colour of hake fillets during storage at 4°C was recorded using a Minolta chromameter (CR-300, Minolta Camera Co., Osaka, Japan) as outlined by (Azlin-Hasim, Cruz-Romero, Morris, Cummins, & Kerry, 2015). Briefly, 10 random areas of the hake surface were measured per sample on each measurement day and the average values of forty readings of CIE *L*-value (lightness), *a*-value (redness) and *b*-value (yellowness) were recorded.

4.3.5.3.4 TBARS (Lipid Oxidation)

Throughout storage, lipid oxidation of the hake fillets was measured using the 2-thiobarbituric acid (TBARS) assay as outlined by (Siu & Draper, 1978). The malondialdehyde (MDA) content was calculated using an extinction coefficient of $1.56 \times 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$ and results were expressed in mg MDA kg⁻¹.

4.3.5.4 Microbiological Analysis

In order to obtain a representative sample, 10g of hake flesh muscle was taken, aseptically, from the top and bottom parts of the hake fillet and placed in a sterile stomacher filter bag. Then, 90mL of sterile MRD was added aseptically into the stomacher bag and homogenised for 180s using BA6021 stomacher 400 (Colworth, Bury St. Edmunds, UK). The homogenate was then ten-fold diluted and used for enumeration of total viable counts (TVC), psychrotrophic bacteria, *Pseudomonas* spp., lactic acid bacteria (LAB), total coliforms and *Escherichia coli* (*E. coli*), anaerobe bacteria and Lyngby agar (H₂S producing bacteria). Total viable count and psychotropic bacteria analysis was carried out using the pour-plate method using PCA and stored at 37°C for 48h and 4°C for 1 week respectively. Lactic acid bacteria (LAB) enumeration was carried out using a pour-plate method with an overlay using De Man, Rogosa and Sharpe (MRS) agar and stored at 30°C for 48hr. Anaerobic bacteria was enumerated using spread-plate method on tryptic soy agar (TSA) + 0.6% yeast extract in an anaerobiosis jar for 72h at 30°C. Lyngby iron agar was used to enumerate presumptive *Shewanella putrefaciens* (black colonies) and *Pseudomonas fluorescens* (white colonies) and incubated at 30°C for 72h. *Pseudomonas* spp. was enumerated after 2 days incubation at 30°C on *Pseudomonas* agar base to which CFC 0103 supplement was added using spread-plate method. For the enumeration of total coliforms and *E. coli*, 1mL of the appropriate dilution was placed in duplicate onto the centre of Compact Dry-EC chromogenic plates and incubated at 37°C for 24hr. Blue colonies were counted as *E. coli* and the total coliforms included red and blue colonies, as indicated by the manufacturer.

4.3.6 Statistical Analysis

All data was analysed for means and standard deviations, and analysis of variance. An analysis of variance of data using Multivariate ANOVA methods was carried out using the SPSS 22 for Windows (SPSS Statistical software, IBM Corp., Armonk, NY, USA) software package. A difference between pairs of means was resolved by means of confidence intervals using Duncan tests; the level of significance was set at $P<0.05$.

4.4 Results and discussion

4.4.1 Effect of Surfactant, Surfactant-to-oil ratio and Physical Treatment on the Particle Size Distribution, Polydispersity Index and Zeta Potential of T20 and P127 Nanoemulsions

The effect of the surfactant, SOR and size reducing treatments on the droplet size, polydispersity index (PDI) and zeta potential of T20 and P127 nanoemulsions are shown in Table 4.1. These parameters affected ($P<0.05$), particle size, PDI and zeta potential of the developed oregano nanoemulsions. Overall, non-treated OEO-P127 (1:2) produced the smallest droplet size. The smaller droplet sizes observed for P127 nanoemulsions compared to T20 nanoemulsions may have resulted from the Pluronic® based surfactant possessing two hydrophilic moieties to one lipophilic moiety which may force the oil to interact in a more compact way (Wulff-Pérez, Torcello-Gómez, Gálvez-Ruíz, & Martín-Rodríguez, 2009). Conversely, T20 is a non-ionic surfactant with one hydrophilic moiety and one lipophilic moiety (Donsì, et al., 2016).

Regarding SOR, a decrease ($P<0.05$) in droplet size with increasing SOR was observed, with exceptions being noted for OEO-T20 (2:1) and OEO-T20 (1:1). These results are in agreement with previous findings of Nejadmansouri, Hosseini, Niakosari, Yousefi, &

Golmakani, (2016), which showed that smaller droplet sizes from an increasing SOR are due to the concentration of surfactant effecting the interfacial tension and therefore, the mobility of the oil-water interfaces where the oil droplets are formed. Consequently, at higher surfactant concentrations, more diffusion of surfactant material from the lipid phase into the aqueous phase occurs, promoting the formation of smaller oil droplets at the oil-water boundary. However, concerning the effect of SOR on PDI, a dimensionless measure of the homogeneity of the particle size distribution and stability of the droplet size calculated from the cumulant analysis (Sugumar, Ghosh, Nirmala, Mukherjee, & Chandrasekaran, 2014; Sullivan, Cruz-Romero, et al., 2018), and zeta potential (a measure of the electrical charge around a colloidal or micellular material suspended in a solution), no discernible zeta potential or PDI trends were apparent.

With respect to physical treatments, smaller ($P<0.05$) droplet sizes were produced using tip-sonication or IKA when compared to untreated emulsions (Table 4.1). For untreated emulsions, T20 nanoemulsions has a larger ($P<0.05$) droplet size and PDI compared to IKA and tip-sonicated nanoemulsions. Concerning zeta potentials, OEO- T20 (2:1) was found to have a smaller ($P<0.05$) zeta potential than OEO-T20 (1:1) and OEO-T20 (1:2). This may be due to the lower surfactant composition affecting the interaction of oil and surfactant and therefore, reducing its zeta potential (A. Acevedo-Fani, Salvia-Trujillo, Rojas-Grau, & Martin-Belloso, 2015). Nonetheless, untreated OEO-T20 (1:1) and OEO-T20 (1:2) values remained above $\pm 30\text{mV}$ and are considered as moderately stable micelles and are therefore, electrostatically-stable and less susceptible to agglomeration and destabilisation forces such as van der Waals forces, Brownian motion or particle-particle interactions (Sullivan, Cruz-Romero, et al., 2018).

Table 4.1 Effect of non-treatment, IKA tube drive and tip sonication treatment on the particle size distribution, polydispersity index and zeta potential of oregano nanoemulsions made from Tween 20 and Pluronic 127. ^{a,b} Mean values with different superscripts indicate difference between SOR are significantly different (P<0.05) and ^{A,B} with different superscripts indicate difference between physical treatments are significantly different (P< 0.05).

	Particle Size Distribution (nm)	PDI	Zeta Potensial (mV)
Untreated			
OEO-T20 (2:1)	782.17±591.0 ^{a, A}	0.6432±0.4 ^{a, A}	14.81±15.4 ^{a, A}
OEO-T20 (1:1)	1925±1203.3 ^{b, A}	0.8928±0.2 ^{a, A}	33.8±5.0 ^{ab, A}
OEO-T20 (1:2)	278.97 ± 49.0 ^{b, A}	0.3087±0.0 ^{b, B}	36.15±1.5 ^{b, A}
OEO-P127 (2:1)	199.60±94.7 ^{a, A}	0.2352±0.1 ^{a, A}	14.95±2.4 ^{a, A}
OEO-P127 (1:1)	73.77±34.0 ^{b, B}	0.1132±0.0 ^{b, A}	5.55±1.4 ^{a, A}
OEO-P127 (1:2)	39.97±1.3 ^{b, B}	0.2403±0.0 ^{a, A}	15.033±14.0 ^{b, A}
IKA			
OEO-T20 (2:1)	658.87±214.4 ^{a, B}	0.6223±0.1 ^{a, A}	41.983±3.0 ^{a, A}
OEO-T20 (1:1)	126.45±37.2 ^{b, B}	0.3057±0.1 ^{a, A}	28.433±0.8 ^{ab, A}
OEO-T20 (1:2)	256.63±147.1 ^{b, B}	0.4818±0.4 ^{a, A}	13.567±15.1 ^{b, A}
OEO-P127 (2:1)	56.045±9.9 ^{b, B}	0.1353±0.1 ^{a, A}	13.855±8.2 ^{a, A}
OEO-P127 (1:1)	49.98±11.6 ^{b, B}	0.1160±0.0 ^{b, A}	3.971±3.2 ^{a, A}
OEO-P127 (1:2)	40.30±3.4 ^{b, B}	0.2138±0.1 ^{a, A}	3.126±4.0 ^{b, A}
Tip sonication			
OEO-T20 (2:1)	148.55±8.3 ^{b, B}	0.1789±0.0 ^{b, B}	31.6±7.9 ^{a, A}
OEO-T20 (1:1)	131.22±2.7 ^{b, B}	0.1663±0.0 ^{b, B}	39.35±5.2 ^{ab, A}
OEO-T20 (1:2)	74.63±7.5 ^{b, B}	0.1070±0.0 ^{b, B}	21.13±1.3 ^{b, A}
OEO-P127 (2:1)	74.04±7.5 ^{b, B}	0.1718±0.1 ^{a, A}	8.212±2.3 ^{a, A}
OEO-P127 (1:1)	54.97±0.6 ^{b, B}	0.0675±0.0 ^{b, A}	11.922±3.4 ^{a, A}
OEO-P127 (1:2)	47.38±3.1 ^{b, B}	0.1353±0.0 ^{a, A}	6.13±2.0 ^{b, A}

In relation to untreated P127 nanoemulsions, larger droplet size was observed with the exception of untreated OEO-P127 (1:2) emulsions which were observed to be smallest droplets. No apparent trend was observed in relation to the zeta potential of P127 emulsions; however, all were below the stability threshold of 30mV, indicating poor long-term stability. Nonetheless, other authors Wulff-Pérez, et al. (2009) have reported that due to the central polypropylene glycol (PPO) moiety linking to the OEO droplet and because of its hydrophobicity, two lateral hydrophilic polyethylene oxide (PEO) moieties remain in the aqueous phase, thereby stabilizing the droplets by steric hindrance.

For IKA treatments, both T20 and P127 nanoemulsions had a smaller ($P<0.05$) droplet size compared to untreated emulsions. In particular, IKA treatments significantly reduced the PDI, in comparison to untreated emulsions for T20 nanoemulsions. However, for the zeta potential of T20 nanoemulsions, no apparent trend was observable. Regarding the PDI and zeta potentials of IKA-treated P127 nanoemulsions, no trends were evident. Overall, the lower droplet size and greater monodispersity of IKA-treated hake fillets can be attributed to the rotor-stator principle, where droplet size reduction occurs via the high circumferential speed drawing the emulsion axially into the dispersion head of the tube and then forcing the emulsion radially through the slots and the minimal gaps between the rotor and the stator to produce an extremely strong shear force (Spinelli, et al., 2010).

Regarding tip-sonication treatments, T20 nanoemulsions had smaller ($P<0.05$) droplet size compared to IKA and untreated emulsions. Concerning PDI results for T20 nanoemulsions, a reduction ($P<0.05$) in PDI occurred compared to both untreated and IKA-treated nanoemulsions; however, in relation to zeta potentials, no trend was apparent. This may perhaps be due to the droplet size reduction mechanism of sonication which

occurs via a high energy wave with a frequency greater than 20Hz which creates rapidly collapsing bubbles in the aqueous solution, thereby generating a high pressure gradient and high velocity in the liquid which results in an internal shear force that will break up the coarse O/W emulsion into a nanoemulsion (Ghosh, Mukherjee, & Chandrasekaran, 2013). For P127 nanoemulsions, no difference ($P>0.05$) in droplet size was observed compared to untreated or IKA-treated emulsions and no apparent effect on PDI or zeta potential was discerned.

Additionally, the emulsification process is also affected by other factors such as the viscosity of the disperse oil phase with respect to the viscosity of the continuous aqueous phase: the closer to unity is the ratio of dispersed phase to continuous phase viscosity, the more efficient is the mechanical emulsification process (Donsì, et al., 2016). Moreover, other factors contribute to control achievable emulsion size, such as the kinetics of surfactant adsorption onto the O/W interface (Donsì, et al., 2016).

4.4.2 AFM analysis of oregano essential oil nanoemulsions

The morphological and topographical features of tip-sonicated T20 and P127 nanoemulsions were assessed due to their beneficial droplet size and monodispersity (Figure 4.1). Overall, it was observed that T20 emulsions were larger than P127 emulsions; however, both were observed to have a spherical micellular morphology which was in agreement with a number of other studies (Ma, et al., 2016)(Ghosh, et al., 2013).

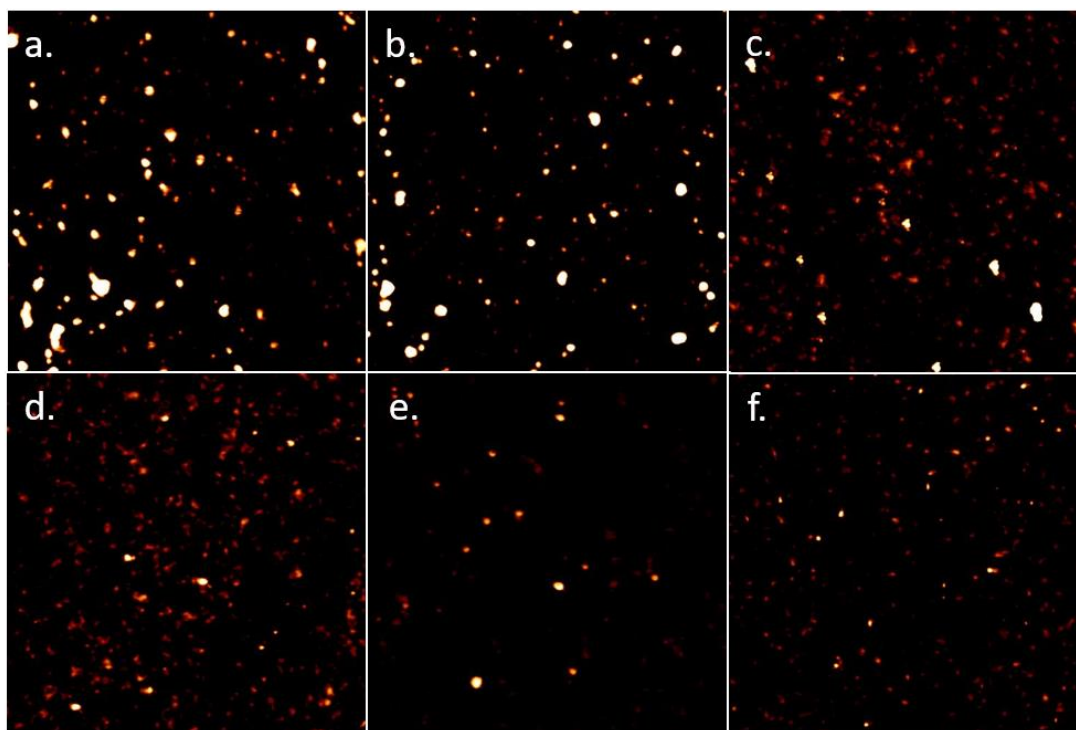


Figure 4.1 AFM topography ($2 \times 2 \mu\text{m}$) of OEO-T20 (2:1) (a.), OEO-T20 (1:1) (b.), OEO-T20 (1:2) (c.), OEO-P127 (2:1) (d.), OEO-P127 (1:1) (e.), and OEO-P127 (1:2) (f.).

Droplet size analysis from AFM showed that the droplet diameters for tip-sonicated T20 nanoemulsions was 96.47, 75.45 and 70.13 nm for OEO-T20 (2:1), OEO-T20 (1:1), and OEO-T20 (1:2), respectively. Regarding P127 nanoemulsions, particle size was observed to be 71.87, 69.80, and 50.47 nm for OEO-P127 (2:1), OEO-P127 (1:1), and OEO-P127 (1:2), respectively. The smaller sizes observed from AFM analysis, in comparison to hydrodynamic measurements, may perhaps be as a result of the hydrophilic moiety of the surfactant polymer, which is encapsulating the oil, deswelling in the absence of water, thereby reducing overall droplet size. Conversely, for OEO-P127 (1:2), the increased droplet size compared to hydrodynamic measurements may perhaps be due to the

interaction of the AFM tip and the nanoemulsions distorting the scanned image (Akbas, et al., 2018).

4.4.3 Antimicrobial activity of developed nanoemulsions

A minimum inhibition concentration (MIC) assay was carried on both T20 and P127 nanoemulsions to assess the effect of the surfactant and SOR on the antimicrobial effect against common Gram-positive (*S. aureus* and *B. cereus*) and Gram-negative (*E. coli* and *P. fluorescens*) food-associated microorganisms (Figure 4.2). Initially, the surfactants were formulated without any antimicrobial oil to determine any inherent surfactant antimicrobial activity and no antimicrobial activity was observed (data not shown). Independent of the SOR used, T20 nanoemulsions were found to have greater antimicrobial activity than P127 nanoemulsions. This may perhaps be due to intrinsic physicochemical properties of the surfactant, as T20 is a small non-ionic surfactant molecule which may allow for more rapid release of the EO; however, P127 is a polymeric surfactant and may slow and impede the release of EO's due to its long chain structure which may more effectively encapsulate the EO.

With respect to the efficacy against pure cultures of *P. fluorescens*, *E. coli*, *B. cereus* and *S. aureus*, for T20 nanoemulsions, no difference ($P>0.05$) in antimicrobial activity against pure cultures of *P. fluorescens*, *E. coli*, *B. cereus* and *S. aureus* were observed for OEO-T20 (2:1) and OEO-T20 (1:1); however, lower ($P<0.05$) antimicrobial activity against cultures of *P. fluorescens*, *E. coli*, and *S. aureus* for OEO-T20 (1:2) was observed. Similarly, for P127 nanoemulsions, no difference ($P>0.05$) in antimicrobial activity against pure cultures of *P. fluorescens*, *E. coli*, *B. cereus* and *S. aureus* were observed for OEO-P127 (2:1) and OEO-P127 (1:1); however, a lower ($P<0.05$) antimicrobial activity

against cultures of *P. fluorescens*, *E. coli*, and *S. aureus* for OEO-P127 (1:2) was observed. Therefore, the most susceptible bacteria to T20 or P127 nanoemulsions, independent of SOR or surfactant type, was *B. cereus*, and this finding is in agreement with other scientific reports (Topuz, et al., 2016), who report that EO's have more antimicrobial activity against Gram-positive than Gram-negative bacteria. The greater susceptibility of Gram-positive than Gram-negative bacteria to EO nanoemulsions has been widely reported and is believed to be associated with the lipophilic ends located on lipoteichoic acid in the cell membrane of Gram-positive bacteria, thereby enabling the penetration of hydrophobic EO constituents into the internal cell structure (Tongnuanchan & Benjakul, 2014). Conversely, the reduced susceptibility of Gram-negative bacteria was attributed to the role of extrinsic membrane proteins and cell membrane lipopolysaccharides, thereby limiting the diffusion of hydrophobic EO compounds into the microorganism (Tongnuanchan, et al., 2014). The antimicrobial mechanism of EO action is not fully understood; however, it has been suggested that the mechanism may occur from a synergistic action of the secondary metabolites such as p-cymene, thymol, and carvacol present in oregano EO's (Hyldgaard, et al., 2012). In particular, carvacrol can disintegrate outer membranes of Gram-negative bacteria, while in Gram-positive bacteria the membrane permeability is altered, thereby allowing permeation of cations like H^+ and K^+ (Hyldgaard, et al., 2012) and p-cymene. While such chemicals are not inherently antimicrobial, they have a high affinity for bacterial cell membranes where they can substitute themselves into the cell membrane, subsequently altering the bacteria's physiological barrier properties and facilitating easier access for other more potent antimicrobial compounds into the cell (Burt, 2004). Furthermore, the

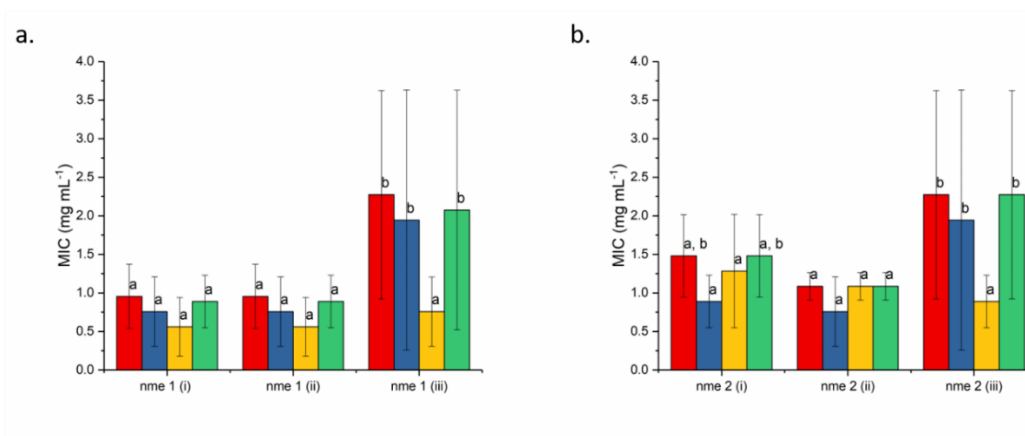


Figure 4.2 Minimum Inhibition Concentration of (a.) OEO-T20 (2:1), OEO-T20 (1:1), and OEO-T20 (1:2) and (b.) OEO-P127 (2:1), OEO-P127 (1:1), and OEO-P127 (1:2). ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P<0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P<0.05$).

shape of the bacteria has also been reported to affect the antimicrobial efficacy of EO's, as rod-shaped cells have been reported to be more sensitive to antimicrobial substances than coccoid-shaped cells (Sullivan, Azlin-Hasim, et al., 2018). Moreover, when compared to pure EO results reported previously by Rodríguez-Calleja, et al. (2015), nanoemulsions of OEO prepared in this study had greater antimicrobial activity. This may perhaps be due to a combination of factors such as: an increased surface area and passive transport through the outer cell membrane, thereby improving interaction with cytoplasmic membranes (Donsì, et al., 2016), through the fusion of the emulsifier droplets with the phospholipid bilayer of the cell membrane, consequently stimulating the targeted release of the EO at the desired sites (Li, et al., 2015), or via the electrostatic interaction

of positively-charged nanoemulsion droplets with negatively-charged microbial cell walls increases the concentration of essential oils at the site of action (Lu, et al., 2018).

4.4.5 Application of size-controlled oregano nanoemulsions on Hake fillets

Due to the previously outlined antimicrobial and physical properties, tip-sonicated OEO-T20 (1:1) nanoemulsions were chosen as the most suitable for aerosolisation treatments.

4.4.5.1 Proximal Composition

The proximal composition of hake fillets caught in the Irish Sea with and without antimicrobial treatment are shown in Table 4.2. No difference ($P>0.05$) with respect to antimicrobial treatment used on the protein, ash, fat and moisture content of hake fillets was observed. Moreover, the proximal composition of hake fillets are in agreement with (Roncarati, et al., 2012) who found that Hake caught in the south Tyrrhenian sea had a 79.9 ± 1.0 , 17.9 ± 0.7 , 1.2 ± 1.7 and $1.5 \pm 0.2\%$ moisture, protein, lipid and ash content, respectively. Nonetheless, variation in proximal analysis has been attributed the variation

Table 4.2 Proximal composition of control (■) and OEO-T20 (1:1) (■) treated hake fillets.

^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P<0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P<0.05$).

	Protein (%)	Ash (%)	Fat (%)	Moisture (%)
Control	17.57±0.6 ^a	1.07±0.1 ^a	0.90±0.3 ^a	79.73±0.5 ^a
nme 1 (ii)	17.17±0.9 ^a	1.09±0.1 ^a	0.75±0.3 ^a	80.09±0.7 ^a

in the composition of hake fillets to environmental and physiological factors including: the nutrition, catching season (spawning cycles), sexual variation, fish size, living area, as well as the other environmental conditions (Dominguez-Petit, Saborido-Rey, & Medina, 2010).

4.4.5.2 pH of Hake fillets during storage

The pH of control and OEO-T20 (1:1) treated vacuum skin packed hake fillets stored at 4 °C are shown in Figure 4.3. Independent of the type of treatment used, the pH values of the hake fillets increased significantly ($P<0.05$) throughout storage until day 6. While the pH was found to be lower for OEO-T20 (1:1) treated fillets than untreated control hake fillets, with results not being significant ($P>0.05$). Conversely, Quitral, et al. (2009) reported that incorporating oregano extracts during the chilled storage of Chilean jack mackerel (*Trachurus murphyi*) reduced ($P<0.05$) the pH of the fish muscle. Nevertheless, the pH of the hake fillets herein did not surpass a pH of 7.0 which is considered as the acceptability limit as above this pH the hake fillet organoleptic properties deteriorate due to the dissociation of carbonic acid and an accumulation of undesirable alkaline compounds such as ammonia and trimethylamine (TMA), which primarily arise as a result of increased microbial action from longer storage times (Garcia-Soto, et al., 2013; Volpe, et al., 2015). However, variation in the initial pH of hake fillets can occur depending on the species, season, diet, level of activity or stress during the catch as well and the type of muscle for consumption. (Ocano-Higuera, et al., 2011).

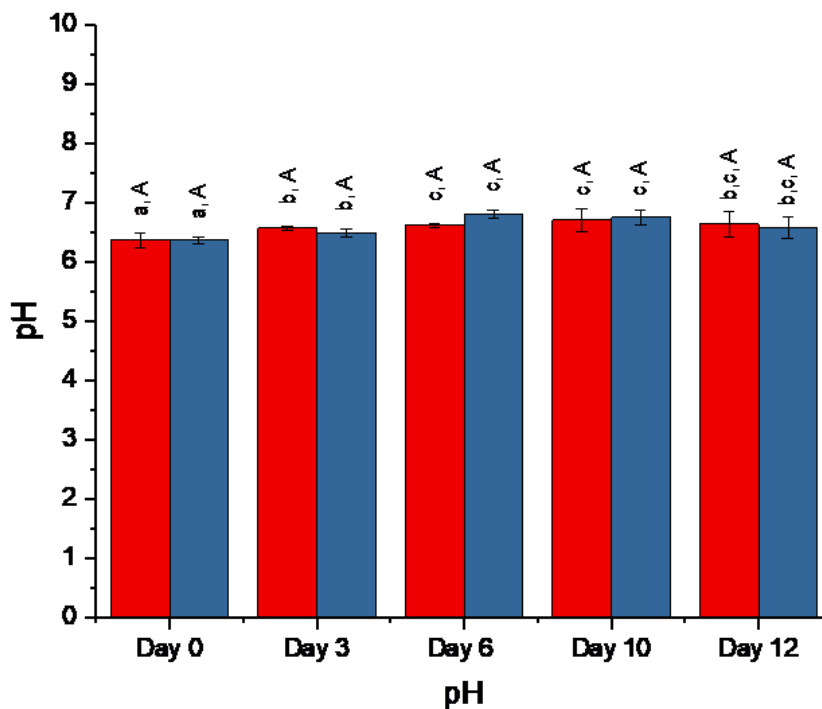


Figure 4.3 pH of control (■) and OEO-T20 (1:1) (■) treated hake fillets. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P<0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P<0.05$).

4.4.5.3 Colour of Hake fillets during storage

The effect of control or OEO-T20 (1:1) treatments on the CIE L -value (lightness), a -value (redness), b -value (yellowness) and whiteness index (WI) of hake fillets can be seen in Table 4.3. During storage, lightness (L -values) were found to increase ($P<0.05$), independent of the treatment used until day 9, after which the lightness begins to decrease. With respect to OEO-T20 (1:1) treated hake fillets, it was observed that these hake fillets differed ($P<0.05$) in comparison to untreated control hake fillets. Concerning redness (a -values), independent of the treatment used, the a -values decreased ($P<0.05$) after day 9.

Moreover, compared to control samples, OEO-T20 (1:1) treated hake fillets had higher ($P<0.05$) a -values.

Table 4.3 Colour of control (■) and OEO-T20 (1:1) (■) treated hake fillets. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P<0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P<0.05$).

	Control				nme 1 (ii)			
	L	a	b	WI	L	a	b	WI
Day 0	59.28±1.1 a, AB	-1.39±0.1 a, A	-1.5±0.3 a, A	59.61±4.1 a,A	57.25±1.0 a, BC	-1.04±0.2 a, AB	-1.7 ± 0.4 a, B	59.23±2.2 ^a ABC
Day 3	59.25±0.9 b, AB	-0.99±0.2 ab, A	-1.06±0.3 b, A	59.46±6.1 a,A	57.94±0.9 b, BC	-1.02±0.1 ab, AB	-1.19±0.5 b, B	58.44±3.2 ^a ABC
Day 6	61.73±0.8 c, AB	-0.77±0.2 ab, A	-0.04±0.5 c, A	59.54±3.5 a,A	60.72±1.1 c, BC	-1.29±0.2 ab, AB	-0.01±0.5 c, B	56.88±2.9 ^a ABC
Day 9	62.59±1.2 d, AB	-1.67±0.4 b, A	-0.58±0.4 d, A	59.31±3.4 a,A	63.16 ± 0.77 ^d , BC	-0.44±0.9 b, AB	1.5±0.6 ^d B	57.35±3.0 ^a ABC
Day 12	60.69±1.0 b, AB	-0.96±0.2 b, A	-0.95±0.4 c, A	60.65±0.9 a,A	60.71±0.7 b, BC	-0.23±0.3 b, AB	1.35±0.5 c, B	55.9±1.8 ^a ABC

Overall, hake fillets have relatively low redness values due to naturally low concentration of “redness” increasing pigments such as carotenoids and/or hemopigments (Sanchez-Zapata, Perez-Alvarez, Fernandez-Lopez, & Barber, 2010). With respect to yellowness (b -values), an increase ($P>0.05$) was observed up to day 9, independent of the treatment used. For b -value of treated hake fillets, it was observed that untreated control hake differed ($P<0.05$) to OEO-T20 (1:1) hake fillets and the increase in b -values for control

hake may be associated with the increased oxidation and formation of metmyoglobin (Ghaderi-Ghahfarokhi, Barzegar, Sahari, & Azizi, 2016). As regards the WI of control and OEO-T20 (1:1) treated hake fillets, independent of treatment, no significant differences were observed throughout the storage period. However, differences ($P<0.05$) were observed between control and OEO-T20 (1:1) treated hake fillets. The difference in WI with respect to treatments may be due to the effect of the antimicrobial coating affecting the WI of hake fillets.

4.4.5.4 TBARS of Hake fillets during storage

The lipid oxidation of control and OEO-T20 (1:1) treated hake fillets was shown in Figure 4.4. Independent of the treatment used, TBARS values decreased significantly ($P<0.05$); however, no significant ($P>0.05$) difference between treatments was observed. Nonetheless, untreated control hake fillets were found to have a higher TBARS than OEO-T20 (1:1) treated hake fillets. The decrease in TBARS may be as a result of the reaction between malondialdehyde and components of the fish muscle such as proteins, amino acids and glycogen which will decrease the amount of malondialdehyde present (Shahbazi, et al., 2018). Moreover, OEO based materials have been widely reported to have strong antioxidant activity which can “mop up” up lipid oxidation initiators; therefore, reducing lipid oxidation pathways (Ojagh, Rezaei, Razavi, & Hosseini, 2010; Shahbazi, et al., 2018; Yang, et al., 2016). The antioxidant mechanism of OEO was

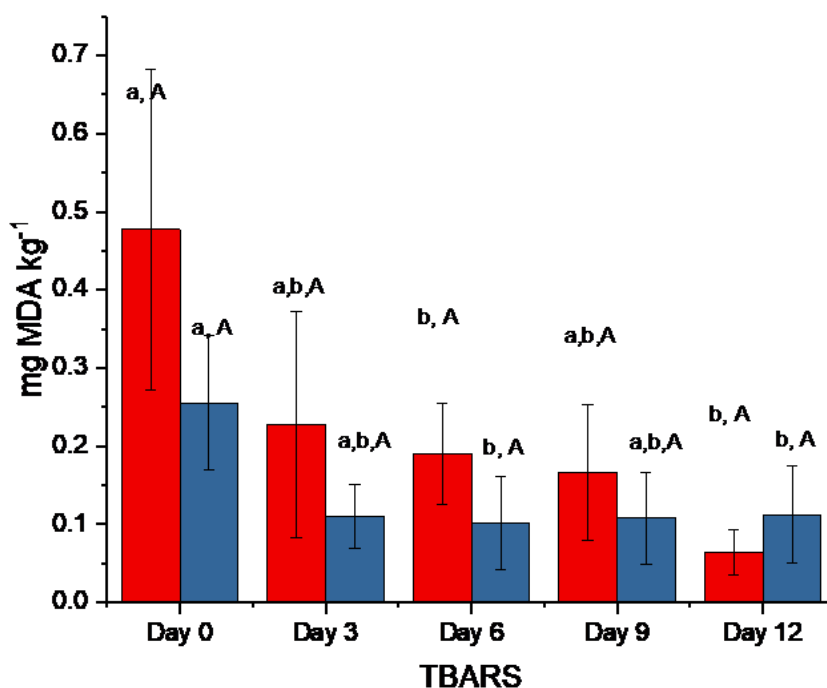


Figure 4.4 TBARS of control (■) and OEO-T20 (1:1) (■) treated hake fillets. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P < 0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P < 0.05$).

ascribed to its radical capturing and metal-ion chelation ability which can capture chain-carrying lipid peroxyl radicals associated with lipid

oxidation and quench their propagation through a radical-radical reaction (Jouki, Yazdi, Mortazavi, Koocheki, & Khazaei, 2014; Nieto, Jongberg, Andersen, & Skibsted, 2013). In addition to the antimicrobial treatments used, VSP has been shown to reduce the effect of lipid oxidation, due to negligible CO₂ to initiate this process in VSP systems (Perez-Alonso, Aubourg, Rodriguez, & Barros-Velazquez, 2004; Rodrigues, et al., 2016).

Nonetheless, independent of the treatment used and throughout storage, the TBARS remained below the 1.5 mg MDA kg⁻¹ limit beyond which fish will normally develop objectionable odours/tastes (M. Cruz-Romero, Kelly, & Kerry, 2008); however, Ojagh, et al. (2010) have suggested that this limit may even be as high as 5 mg MDA/kg for fresh fish. The results reported herein were in agreement with Garcia-Soto, et al. (2013) who found that lipid oxidation is not a known or important damage pathway during chilled storage of lean white fish species such as hake fillets.

4.4.5.5 Microbiological analysis of Hake Fillets

From a microbiological perspective, the upper acceptable limit for the TVC of fish products is 7 log CFU g⁻¹ (Carrion-Granda, et al., 2018). However, in this study, a TVC value of 6 log CFU g⁻¹ was set as the maximum limit of acceptability. The effect of OEO-T20 (1:1) treatments in combination with VSP stored at 4°C on the microbial counts of TVC, anaerobes, psychrotrophs, *Pseudomonas* spp., H₂S-producing bacteria, LAB, *E. coli*, and coliforms are shown in Figure 4.5. A reduction ($P<0.05$) in the populations of TVC, anaerobic and coliform bacteria was observed for hake fillets treated with OEO-T20 (1:1) compared to untreated control hake fillets. While higher microbial populations of LAB, *Pseudomonas* spp., psychrotrophs, *E. coli*, and H₂S-producing bacteria were observed in untreated control treatments compared to OEO-T20 (1:1) treated hake fillets; however, these differences were not found to be statistically significant ($P>0.05$).

Regarding TVC, the initial bacterial count for control hake fillets was 4.40 log CFU g⁻¹, while the TVC for hake fillets treated with OEO-T20 (1:1), was 4.16 log CFU g⁻¹. However, the initial microbial count was relatively high compared to the initial TVC of untreated hake fillets caught off the Galician Atlantic coast (North-Western Spain) which

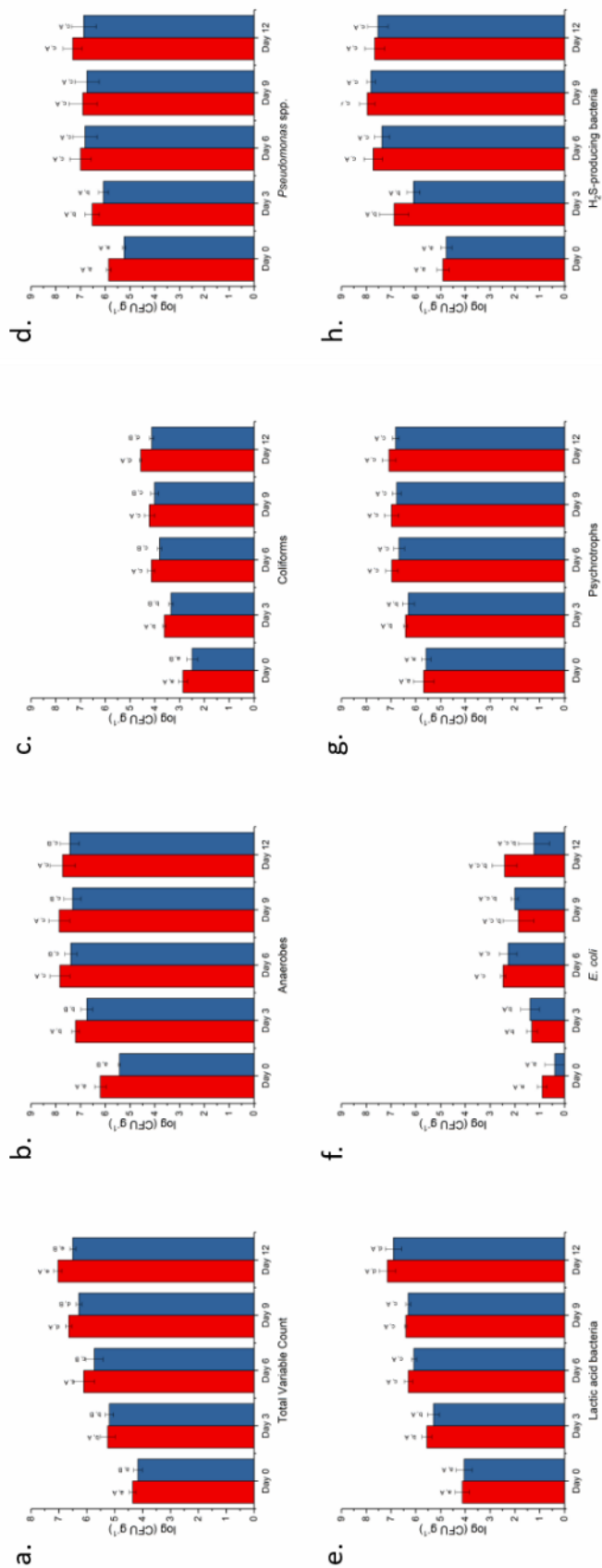


Figure 4.5 The microbiological count of (a) TVC, (b.) anaerobic bacteria (c) coliforms, (d) *Pseudomonas* spp., (e.) LAB, (f.) *E. coli*, (g.) psychrotrophic bacteria, and (h.) H₂S-producing bacteria for control (■) and OEO-T20 (1:1) (■) treated hake fillets stored at 4°C under vacuum skin packaging over the 12 day storage period. ^{a,b} Mean values with different superscripts indicate difference between storage days are significantly different ($P < 0.05$) and ^{A,B} with different superscripts indicate difference between antimicrobial treatments are significantly different ($P < 0.05$).

was 2.46 log CFU g⁻¹ (Garcia-Soto, et al., 2013). The higher initial TVC of hake fillets observed herein may perhaps be due to poor handling practises post-mortem such as: manual processing, handling, storage conditions, the skinning process, the time taken to reach the fishery plant, and the hygiene condition of the fishery/processing plant during harvesting (Carrion-Granda, et al., 2018; Günlü & Koyun, 2013). Untreated control hake fillets reached the set limit of acceptability on day 6; however, hake fillets treated with OEO-T20 (1:1) reached the acceptability limit after 8.5 days of storage. This represents an extension ($P<0.05$) of the shelf-life of OEO-T20 (1:1) treated hake fillets upon untreated control fillets of up to 50 %.

With respect to anaerobic microbial population, a difference ($P<0.05$) between untreated control and OEO-T20 (1:1) treated hake fillets was observed (Figure 4.5 b.). The initial anaerobic bacterial count was 6.20 and 5.45 log CFU g⁻¹ for untreated control and OEO-T20 (1:1) treated hake fillets, respectively. The initial microbial count of anaerobic bacteria observed herein was high, as other authors such as, (Perez-Alonso, et al., 2004), who packaged and stored Atlantic pomfret (*Brama brama*) using VSP at 4 °C found that the initial anaerobe colonies was 4.11 log CFU g⁻¹. The high initial loadings may perhaps be due afore mentioned handling conditions. Regarding coliforms, a difference ($P<0.05$) between untreated control fillets and OEO-T20 (1:1) treated fillets was observed (Figure 4.5 c.). Low initial counts of coliform bacteria have been reported elsewhere by Volpe, et al. (2015) who coated rainbow trout fillets with a carrageenan enriched with essential lemon oil. These result indicate good hygiene conditions in the fishery plant (Shahbazi, et al., 2018).

Regarding psychrotrophic bacteria, *Pseudomonas* spp., H₂S-producing bacteria, LAB bacteria, and *E. coli* the results of this study indicate that untreated control had higher microbial populations compared to OEO-T20 (1:1) treated hake fillets; however, this was not statistically significant ($P>0.05$) (Figure 4.5 d. – h.). Nonetheless, the microbial counts for psychrotrophic bacteria, *Pseudomonas* spp., LAB bacteria, and *E. coli* were all under their respective upper limit of acceptability. With respect to Gram-positive LAB bacteria, they are regarded as the most resistant Gram-positive bacteria to EO's and their increased resistance to OEO antimicrobial treatments has been attributed to their greater ability to deal with conditions of osmotic stress and their effective response to K⁺ efflux caused by EO's (Jouki, et al.), #0}.

4.5 Conclusion

Results from this study indicate that factors such as surfactant type, SOR and physical treatments significantly affect the droplet size and PDI of T20 and P127 nanoemulsions; however, the smallest nanoemulsions did not produce the greatest antimicrobial affect; therefore, suggesting that other compositional factors such as surfactant type used or SOR also need to be considered in choosing the appropriate formation parameters. Tip sonicated OEO-T20 (1:1) were chosen as the most suitable nanoemulsions for aerosolisation treatments on hake fillets due to their small and monodisperse droplet size and their strong antimicrobial activity. Results indicated that the microbiological and physiochemical properties of hake fillets aerosolised with OEO-T20 (1:1) as part of a hurdle strategy was found to extended ($P<0.05$) the shelf life of hake fillets by up to 50 % compared to untreated control hake fillets. Overall, this study has indicated that oregano nanoemulsions with high monodispersity and small droplet size are suitable materials for

aerosolisation applications on hake fillets as part of a hurdle strategy to extend their shelf life. Moreover, aerosolisation treatments have many favourable attributes including; requiring small volumes of antimicrobial solutions, good coverage, facile application, relative cheap cost, minimal impact on physiochemical parameters such as proximal analysis, and fulfilling clean label criteria and therefore, could be used to treat a diverse range of high value or perishable food products including; fruits, vegetables, chicken, beef and seafood.

4.6 References

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Chapter 5: Development of a Simple Method to Deliver Antimicrobial Oil into a Food System: Possible Attachment to Food Packaging?

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This Chapter is in preparation for submission to an academic journal.

5.1 Abstract

Essential oils have been proposed as suitable antimicrobials in food packaging. However, binding these to a surface is challenging. The objective of this study was to develop a simple method to attach an antimicrobial essential oil support material to a surface via a high surface area carrier which could be bound to the surface chemically. Hence, SBA-15 (a mesoporous silica) was functionalized with (3-aminopropyl)triethoxysilane (APTES) via a post-synthetic reaction and then attached to a 3-glycidyloxypropyltrimethoxysilane (GPTS) modified surface. The functionalisation of SBA with APTES was assessed using elemental analysis, TGA, N₂ adsorption, FTIR while the functionalisation to GPTS modified surface was characterised using XPS, dynamic contact angle, AFM, ellipsometry and SEM. Results indicate that SBA functionalised with APTES was successfully bound to the GPTS modified surface. Mesoporous SBA-15 has been proposed as smart delivery device capable of loading and releasing large amounts of antimicrobial oregano essential oil (OEO) and herein the antimicrobial activity of OEO loaded into SBA-15 (SBA-OEO), SBA-APTES, and SBA-APTES attached to a GPTS modified surface loaded with OEO were assessed against common food spoilage microorganisms *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas fluorescens*. Results indicate that functionalization of the SBA-15 with APTES resulted in greater antimicrobial activity than SBA-OEO; however, when attached to a GPTS modified surface no antimicrobial activity was observed. Nonetheless, this study has demonstrated that SBA-APTES was successfully bound to a GPTS modified surface and may find applications in other sectors such as biomedical devices, nutraceuticals etc.

5.2 Introduction

Global food security challenges such as an increasing global population (estimated to reach 9 billion by 2050), increasing rural to urban migration, climate change and the waste of packaged food through microbial decomposition and contamination are putting increased pressure on global food production (Sullivan, et al., 2018). Moreover this is compounded by the estimated 1.3 billion metric tons of edible food produced for human consumption that is either lost or wasted annually throughout the supply chain (FAO, 2011). Therefore, food packaging has a significant role in the food supply chain and it is an integral part of both the food processes and the whole food supply chain with the main aim to protect food from contamination. Recently, extensive research has been carried out on the incorporation of natural antimicrobial materials (NAM's) in packaging materials due to increasing consumer demand for sustainable, fresh-like, minimally processed, and clean label food products (Sullivan, et al., 2018). One such suitable class of NAM's are essential oils (EO's) which are defined by the ISO (2013) as a product obtained by steam distillation from a natural raw material of plant origin. These materials have favourable properties such as good antimicrobial activity, their GRAS (Generally Recognised as Safe) status approved by the Food and Drug Administration (FDA) and an acceptability to the consumer attributable to their historical use as a natural flavouring, therefore, making them suitable to be used in food contact materials. Structurally EO's are made up of a combination of secondary metabolites constituents such as terpenes, terpenoids, and phenylpropanoids. These metabolites such as p-cymene, thymol, and eugenol can synergistically contribute to the antimicrobial activity of EO's via interactions with cell

walls components (Amorati, Foti, & Valgimigli, 2013; Hyldgaard, Mygind, & Meyer, 2012).

Nonetheless, roadblocks in the application of antimicrobial EO's in packaging applications exist such as their hydrophobic character, thermolability, photosensitivity, and strong influence on organoleptic properties (Sullivan, et al., 2018). Moreover, due to these volatile properties, EO's are highly susceptible to autoxidation, isomerisation and thermal rearrangements (Bilia, et al., 2014; Calo, Crandall, O'Bryan, & Ricke, 2015; Turek & Stintzing, 2013). Therefore, strategies such as encapsulation into a support porous siliceous material (Sun, Lu, Qiu, & Tang, 2017) such as Santa Barbara Amorphous (SBA)-15 or Mobil Composition of Matter No. 41 (MCM-41) have been used to protect EO's from environmental stressors while also reducing their negative organoleptic impact and interaction with food components and allowing for a controlled release of the EO (Ruiz-Rico, et al., 2017). In particular, research has been carried out on the encapsulation of EO materials into SBA-15 due to these materials current use in the food sector as catalysts in the synthesis of nutrients, bioactive molecules, in sensor technology and as carriers to design smart delivery systems (Ruiz-Rico, et al., 2017). Moreover, these materials have mechanical and hydrothermal stability (Hernández-Morales, et al., 2012), adjustable nano-pore size (Sun, et al., 2017), ordered pore structure (Maria Chong & Zhao, 2003), large specific surface area ($\sim 1000 \text{ m}^2 \text{ g}^{-1}$) (Maria Chong, et al., 2003) and relatively large void volume (Hernández-Morales, et al., 2012) and are also considered GRAS and are an authorised additive in the European Union under E-551 (Ruiz-Rico, et al., 2017).

SBA-15 materials can readily undergo functionalisation with various organic functional containing groups such as 3-aminopropyltriethoxysilane (APTES) which are covalently linked on the wall of the porous structure via hydrolysis and/or condensation reactions giving rise to an amine modified porous silica while retaining the mesoporous silica's favourable physical properties (Vilarrasa-García, et al., 2014). Moreover, the amine modified SBA can be reacted further with other organo-functional alkoxy silanes such as 3-glycidoxypropyl-trimethoxysilane (GPTS) as the epoxy group can undergo various reactions such as poly-addition to the amine group or hydrolytic ring opening. In addition, the trialkoxysilyl moiety of GPTS can undergo hydrolysis and condensation reactions with terminal –OH groups (Riegel, et al., 1998).

Current methods used in incorporate EO materials include: direct incorporation into the polymeric packaging material (Yang, et al., 2016), coating onto packaging materials surface (Erkan, 2012), and as part of an inherently antimicrobial film forming polymer (such as CS) (Shahbazi & Shavisi, 2018). Moreover, authors have reported on the use of mesospheric silica supports for EO delivery in food applications such as (Park, Barton, & Pendleton, 2011) who loaded natural antimicrobial allyl isothiocyanate into MCM-41 and SBA-15 and found they were antimicrobially active against *Escherichia coli*, *Bacillus cereus*, and *Pichia anomola*. (Ruiz-Rico, et al., 2017) tested the antimicrobial activity of vanillin grafted onto the surface of MCM-41 and reported that a significant ($P<0.05$) reduction of $1.5 \log \text{CFU mL}^{-1}$ in the concentration of *Listeria innocua* in pasteurized skimmed milk.

However, to the best of our knowledge, none have investigated the potential novel strategy of using SBA functionalised with APTES and its covalent attachment to a GPTS modified

surface. This would allow a carrier to be fixed to a surface limiting migration of the particles and allowing high density binding so as to achieve high enough concentrations of OEO to be effective. Therefore, the objectives of this study were to covalently attach SBA functionalised with APTES to GPTS modified surface as a support material for OEO. The synthesised materials were subsequently characterised and their antimicrobial activity assessed.

5.3 Materials and Methods

5.3.1 Materials

Oregano essential oil was purchased from Lionel Hitchens Ltd (Barton Stacey, Hampshire, UK). Mueller-Hinton Broth (MHB), Mueller-Hinton Agar (MHA), and Maximum Recovery Diluent (MRD) were purchased from Oxoid (Basingstoke, UK). *Escherichia coli* (*E. coli*) (NCIMB 11943), *Bacillus cereus* (*B. cereus*) (NCIMB 9373), *Staphylococcus aureus* (*S. aureus*) (NCIMB 13062) and *Pseudomonas fluorescens* (*P. fluorescens*) (NCIMB 9046) were maintained on Tryptic Soy Agar slants until use at 4 °C. Siliceous SBA-15 mesoporous material was purchased from Glantreo, Ireland. Blanket Si substrates were purchased from Sil'tronix, France. Sulphuric acid (ACS reagent 95-98%), 3-Aminopropyltriethoxysilane (99%) (APTES), (3-glycidyloxypropyl)trimethoxysilane (>98%) (GPTS), hydrogen peroxide solution (30%), 2-propanol (CHROMASOLV, for high performance liquid chromatography [HPLC], 99.9%), dimethyl sulfoxide (anhydrous 99.9%) were all purchased from Sigma Aldrich, Ireland. Deionised water was purchased from Acros Organics and was used as necessary.

5.3.2 Functionalisation of SBA-15 with APTES

APTES was grafted to the surface of the SBA-15 by weighing out 1 g of mesoporous silica which was then placed into a 50 ml receiver flask with a magnetic stir bar to which 20 ml of DMSO and 2 ml of APTES was added (dropwise). The reaction was allowed to take place over a 20 h period before being removed, filtered and washed with 20 ml DMSO, 20 ml 2-propanol and finally 3 aliquots of 20 ml of deionised water. The SBA functionalised with APTES (SBA-APTES) was then dried in a vacuum oven 90 °C for 1 h.

5.3.3 Preparation of Si-GPTS

To prepare Si coupons for GTPS attachment, the Si substrate was cut into 1 cm² wafers and placed in a round bottom flask with 80 mL of piranha solution (3:1; H₂SO₄/H₂O₂) for 1 h at 100 °C under constant stirring. Once complete, the hydroxylated Si wafers were removed from piranha solution, washed with deionised water, and dried under a stream of N₂ gas, and placed in sample holders until functionalised within an hour. To attach GPTS onto the hydroxylated Si wafers, the wafers were submersed in a 9 % GTPS in DMSO solution and the reaction was carried out over 20 h at 90 °C. Once complete, the wafers were removed, washed with deionised water and dried using N₂ gas and placed in sample holders until further use.

5.3.4 Attachment of Si-GPTS with SBA-APTES

The SBA mesoporous silica material functionalised with amine terminal group were attached to the wafers functionalised with GTPS epoxide functional group (Figure 5.1). To achieve this, the wafers were submerged in 25 mL of deionised H₂O to which 1 g of SBA-APTES was added. The solution was then allowed to stir for 2 hours at 90 °C where upon the wafers were removed, washed with deionised water to remove unbound SBA-APTES and, dried with N₂ gas.

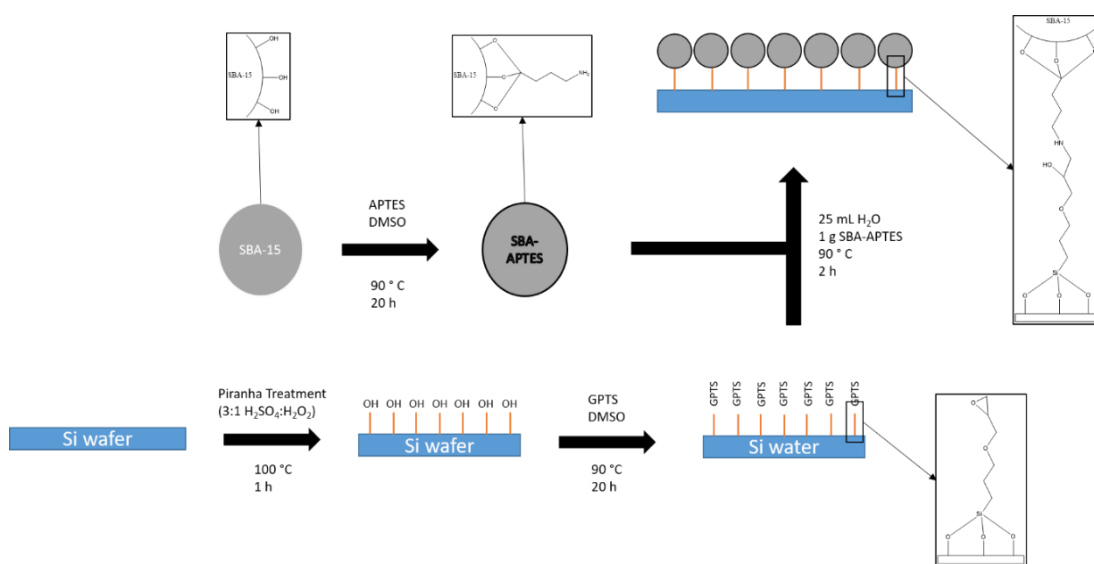


Figure 5.1 Schematic representation of the synthesis of Si-GPTS-APTES-SBA modified surfaces.

5.3.5 Loading of Oregano essential oil into SBA

5.3.5.1 Loading of SBA and SBA-APTES

SBA and SBA-APTES were loaded with OEO by dissolving 1 mL of OEO in 10 mL of absolute ethanol. And was added to either 0.5 g of SBA or SBA-APTES and allowed to dry at room temperature (21 °C) in a partially closed container for 72 h, to ensure full evaporation of ethanol. Samples were weighed before and after loading.

5.3.5.2 Loading of SBA functionalised surfaces

The SBA modified surfaces were loaded with 10 % v/v OEO in ethanol solution via drop casting. Once the solution had evaporated, the wafer was gently washed with sterilised deionised water to remove unabsorbed EO solution.

5.3.6 Characterisation

5.3.6.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was carried out using a Karl Zeiss Ultra Plus field emission SEM with Gemini column. The technique was used to provide detailed visual information on the external surface morphology. A secondary electron detector was used. The samples were placed on carbon tape and then adhered to a stainless-steel stub before being placed in the instrument's chamber. It was operated at 5 keV and various magnifications were used as required.

5.3.6.2 Fourier Transform Infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy (FTIR) analysis of OEO, SBA-15, SBA-OEO and SBA-ATPS-OEO was performed on a Varian 660-IR spectrometer (Varian Resolutions, Varian Inc, Victoria, Australia) using a diamond crystal ATR Golden Gate (Specac). Data were taken with 32 scans at 2 cm^{-1} resolution in a wavenumber range from $4000 - 500\text{ cm}^{-1}$.

5.3.6.3 X-ray Photoemission Spectroscopy (XPS)

X-ray photoelectron spectroscopy was performed under ultra-high vacuum conditions ($<5 \times 10^{-10}$ mbar) on a VG Scientific ESCA-lab Mk II system equipped with a hemispherical analyser using Al K α x-rays (1486.6 eV). The emitted photoelectrons were collected at a take-off angle of 90° from the disks' surface. The analyser pass energy for the survey

scans was 200 eV. The binding energy scale was referenced to the adventitious carbon 1s core level scans at 284.8 eV. Core level scans of Si 2s, C 1s, N 1s and O 1s were examined.

5.3.6.4 N₂ Adsorption-desorption Isotherms

The surface area, pore diameter, pore volume and pore size distribution measurements of the samples were performed based on the sorption technique using the Micromeritics Tristar II surface area analyser (Micromeritics, Norcross, GA, USA). The specific surface area of the samples were calculated using the multi-point Brunauer, Emmett and Teller (BET) method in the relative pressure range $P/P_0 = 0.05-0.3$. The specific pore volume, pore diameter and pore size distribution curves were computed based on the Barrett-Joyner-Halenda (BJH) method. The sorption analysis was carried out at 77 K and each sample was degassed under nitrogen for five hours at 200 °C prior to analysis.

5.3.6.5 Elemental Analysis

Elemental analysis was carried out on SBA-15, SBA-OEO, SBA-ATPES and SBA-APTES-OEO to determine the percentage carbon, nitrogen and hydrogen in the sample. The analysis was performed on Exeter Analytical CE 440 elemental analyser. All samples analysed were carried out in triplicate.

5.3.6.6 Thermal Gravimetric Analysis (TGA–DTG)

In order to evaluate the influence of temperature on the adsorbent stability, the adsorbents were studied by thermal gravimetric analysis. All TG/1st DTG/2nd DTG curves were obtained on a Model TGA 2950 high-resolution thermogravimetric analyzer V5.4a, on a temperature level from 30 to 800 °C with a warming speed of 5 °C min⁻¹ under nitrogen flow.

5.3.6.7 Contact Angle

Dynamic contact angle and surface free energy were calculated from the advancing and receding water contact angles and were recorded on three different regions of each sample as outlined by (Lundy, et al., 2017). Briefly, 60 nL of the liquid was dispensed on the material surface at a flow rate of 5 nL s⁻¹ using a microinjection syringe pump (SMARTouch, World Precision Instruments, Sarasota, FL, USA) with a needle ($\phi = 130$ μm) and images were captured with a monochrome industrial camera (DMK 27AUR0135, The Imaging Source, Bremen, Germany). Contact angles were calculated using a piecewise polynomial fit (ImageJ, ver. 1.46, DropSnake plugin).³¹ The same procedure was used for diiodomethane (CH₂I₂) contact angles. The Surface free energy values were calculated from contact angles of deionized water and diiodomethane the using Owens–Wendt model (Eq 5.1.).

$$\frac{\gamma_{lv}(\cos \theta + 1)}{2} = \sqrt{\gamma_{lv}^D + \gamma_{sv}^D} + \sqrt{\gamma_{lv}^P + \gamma_{sv}^P} \quad (5.1)$$

Surface energy values of H₂O $\gamma_{lv}^D/\gamma_{lv}^P = 21.8/50.8$ mJ m⁻² and CH₂I₂ $\gamma_{lv}^D/\gamma_{lv}^P = 48.5/2.3$ mJ m⁻² were used.

5.3.6.8 Film Thickness

The film thickness of Si-GPTS coated surfaces was obtained from spectroscopic ellipsometry (α -SE J.A. Woollam, Lincoln, NE) at an incident angle of 65 ° and a Cauchy model extrapolation was used to determine the film thickness, n, and k values.

5.3.6.8 Atomic Force Microscopy

Atomic Force Microscope (AFM, Park systems, XE-7, South Korea) measurement on Si-GPTS were performed in non-contact mode with high resolution, silicon micro-cantilever tips. Topographic images were recorded at a resonance frequency of 270-300 kHz.

5.3.6.9 Antimicrobial Assay

In this study, the antimicrobial activity of SBA-OEO and SBA-APTES-OEO against Gram-positive bacteria (*Staphylococcus aureus* (*S. aureus*) (NCIMB 13062) and *Bacillus cereus* (*B. cereus*) (NCIMB 9373)) and Gram-negative bacteria (*Escherichia coli* (*E. coli*) (NCIMB 11943) and *Pseudomonas fluorescens* (*P. fluorescens*) (NCIMB 9046)) were assessed. Before use, all pure culture bacteria were grown for 18 h at 30 °C (*P. fluorescens* and *B. cereus*) or 37 °C (*S. aureus* and *E. coli*) in Mueller-Hinton broth (MHB) (Oxoid, UK) under constant agitation at 170 rpm on an orbital shaker (Innova 2300, New Brunswick™, Germany).

5.3.6.9.1 Minimum Inhibition Concentration (MIC) assay

The antimicrobial activity of SBA and SBA-ATPES loaded with OEO was measured by determining the minimum inhibitory concentration (MIC) against the target microorganisms in a 96 flat bottom well tissue culture microplates (Sarstedt Inc., NC, USA) according to the NCCLS (2000) broth microdilution method as described by (Cruz-Romero, Murphy, Morris, Cummins, & Kerry, 2013). Bacterial strains were cultured overnight at the appropriate temperature and adjusted to a final density of 10⁵ CFU/mL using maximum recovery diluent, and used as an inoculum within 15 min of preparation. Briefly, 100 µL of double-strength MHB (2XMHB) was added to each well in rows A to F, 200 µL of adjusted bacterial culture suspension was added to row H in columns 1–11

and 200 μL of sterile 2XMHB was added to column 12. In each well of row G, 150 μL of SBA-OEO or SBA-APTES-OEO were dispensed in sterile distilled water and a threefold serial dilution was performed by transferring 50 μL of antimicrobial solutions from row G into the corresponding wells of row F through to row B. After mixing, 50 μL of the resultant mixture on row B was discarded. Finally, using a 12-channel electronic pipette (Model EDP3-Plus, Rainin, USA) 15 μL of the tested microorganisms was pipetted from each well in row H into the corresponding wells in row A followed by rows B to G. Positive (Row A) and negative growth controls (Column 12) were included in each assay plate. The inoculated plates were incubated in a wet chamber for 24 h at 30 °C (*P. fluorescens* and *B. cereus*) or 37°C (*E. coli* and *S. aureus*). After incubation, resazurin salt (0.015 %) was added to wells A – F (30 μL per well), and incubated for an additional 2 h. Columns with no colour change (resazurins blue colour remained unchanged if no microbial growth; however, will turn pink if microbial growth occurs) were scored as above the MIC value. The lowest concentration showing inhibition of growth was considered to be the MIC for the target microorganisms. The test was repeated in triplicate.

5.3.6.9.2 Modified Disk Diffusion Assay

The antimicrobial activity of SBA functionalised surfaces was assessed using a modified agar diffusion method where Mueller-Hinton agar (MHA) plates were swabbed with the target microorganism ($\sim 10^5$ CFU ml^{-1}). The SBA functionalised surfaces substrates were then placed in the middle of the inoculated agar plates and incubated for 24 h at 30 °C for *P. fluorescens* and *B. cereus* and at 37 °C for *S. aureus* and *E. coli*. Positive control used a streptomycin antibiotic disc, while unloaded SBA functionalised surfaces were tested

as the negative control. The inhibition zone around the substrate indicated the antimicrobial activity against the target microorganism and was measured using an electronic calliper (Model ECA 015D Moore & Wright, Paintain tools Ltd., Birmingham, UK) in millimetres.

5.4. Results and discussion

5.4.1 Functionalisation of SBA-15 with APTES

The N₂ adsorption/desorption isotherms and total pore volume (V_{total}) of the SBA and SBA-APTES are shown in Figure 5.2. The BET surface area (S_{BET}) was also calculated and found to be 436.33 m² g⁻¹ (data not shown). The un-functionalised SBA-15 displays a type IV isotherm with H1 hysteresis and a sharp increase in volume adsorbed, a characteristic of highly ordered mesoporous materials (Maria Chong, et al., 2003). Moreover, the BJH pore size distribution curves were calculated from the desorption isotherms of SBA- 15 shown in Figure 5.2. The pore sizes (D_{BJH}) estimated from the peak positions of the BJH pore size distribution curves measured from both the adsorption and desorption isotherms and were found to be 27 Å.

The FTIR spectrum of SBA-15, APTES, and SBA-APTES were used to assess the functionalisation of SBA with APTES and are shown in Figure 5.3. The FTIR spectra of unmodified SBA-15 (Figure 5.3 a.) showed major peaks at: 1000 – 1130 cm⁻¹ from symmetrical and asymmetrical stretching of the Si-O-Si backbone of SBA (Maria Chong, et al., 2003), 3400 cm⁻¹ from the silanol groups that cover the surface of SBA and are cross hydrogen bonding with adsorbed water (Vilarrasa-García, et al., 2014), and at 3740 cm⁻¹ which corresponds to the symmetric stretching of terminal Si – O – H. With respect

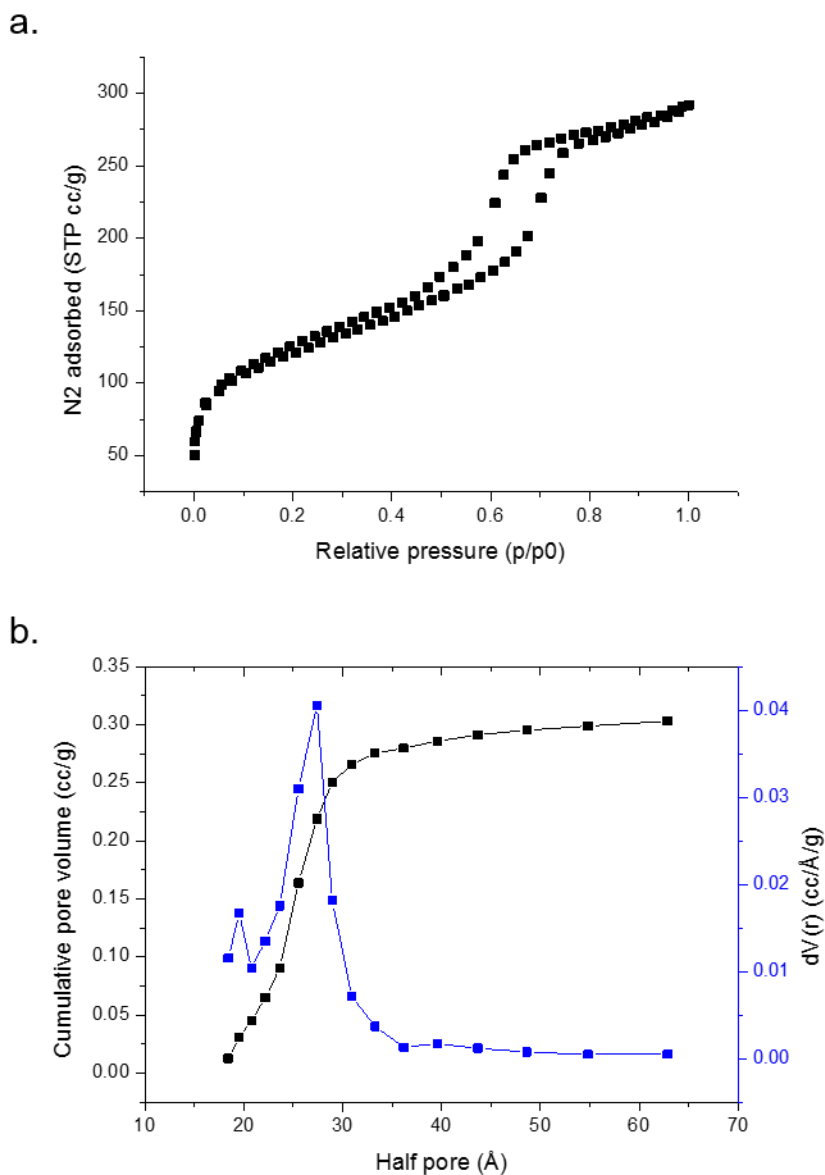


Figure 5.2 Nitrogen adsorption/desorption isotherms at 77 K for SBA-15 (a.), BJH pore size distribution curves of SBA-15 (b.)

to APTES, the FTIR spectra shows characteristic peaks at: $1000 - 1130 \text{ cm}^{-1}$ which are characteristic of symmetrical and asymmetrical stretching of Si-O groups, 1388 cm^{-1} which is attributed to stretching C – N, and at 2884, 2962 and 2974 are attributed to stretching C – H bonds. Moreover a C – O was observed at 1070 cm^{-1} and at 1600 cm^{-1}

due to the H bending on the N of amine group. Regarding SBA-APTES, several new peaks emerge compared to SBA-15 due the presence of APTES at: 2927 and 2857 cm^{-1} greater intensity of the peaks was observed due to a vibrational stretching C – H groups from the APTES, at $3300 - 3360\text{ cm}^{-1}$ which is attributed to a combination of asymmetrical and symmetrical NH_2 stretches and at 1646 cm^{-1} due to the H bending on the N of NH_2 . When comparing the spectra of SBA and ATPES to SBA-ATPES, peak characteristic of both SBA and APTES were observed. Furthermore the disappearance of

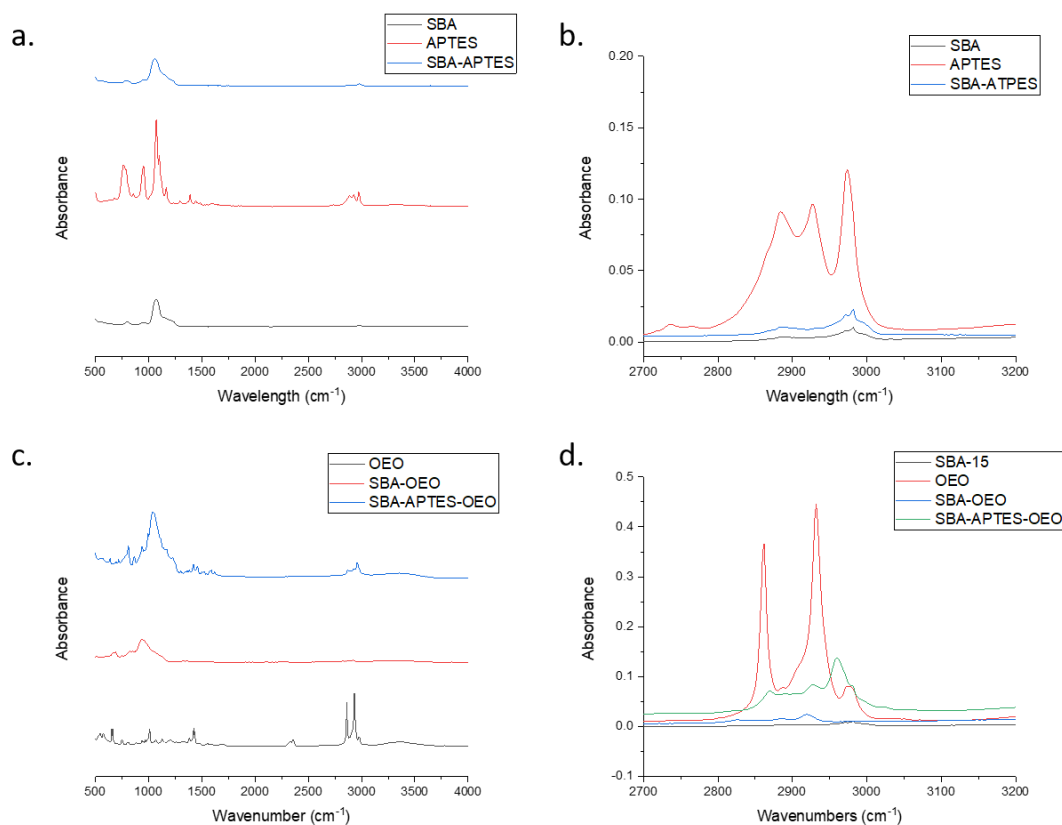


Figure 5.3 FTIR spectra of SBA, APTES, and SBA-APTES (a. – b.) and of OEO, SBA-OEO, and SBA-APTES-OEO (c. – d.).

the terminal Si – OH stretch at 3740 cm^{-1} would suggest that the ethoxy group from the ATPES has bound to the surface of the SBA.

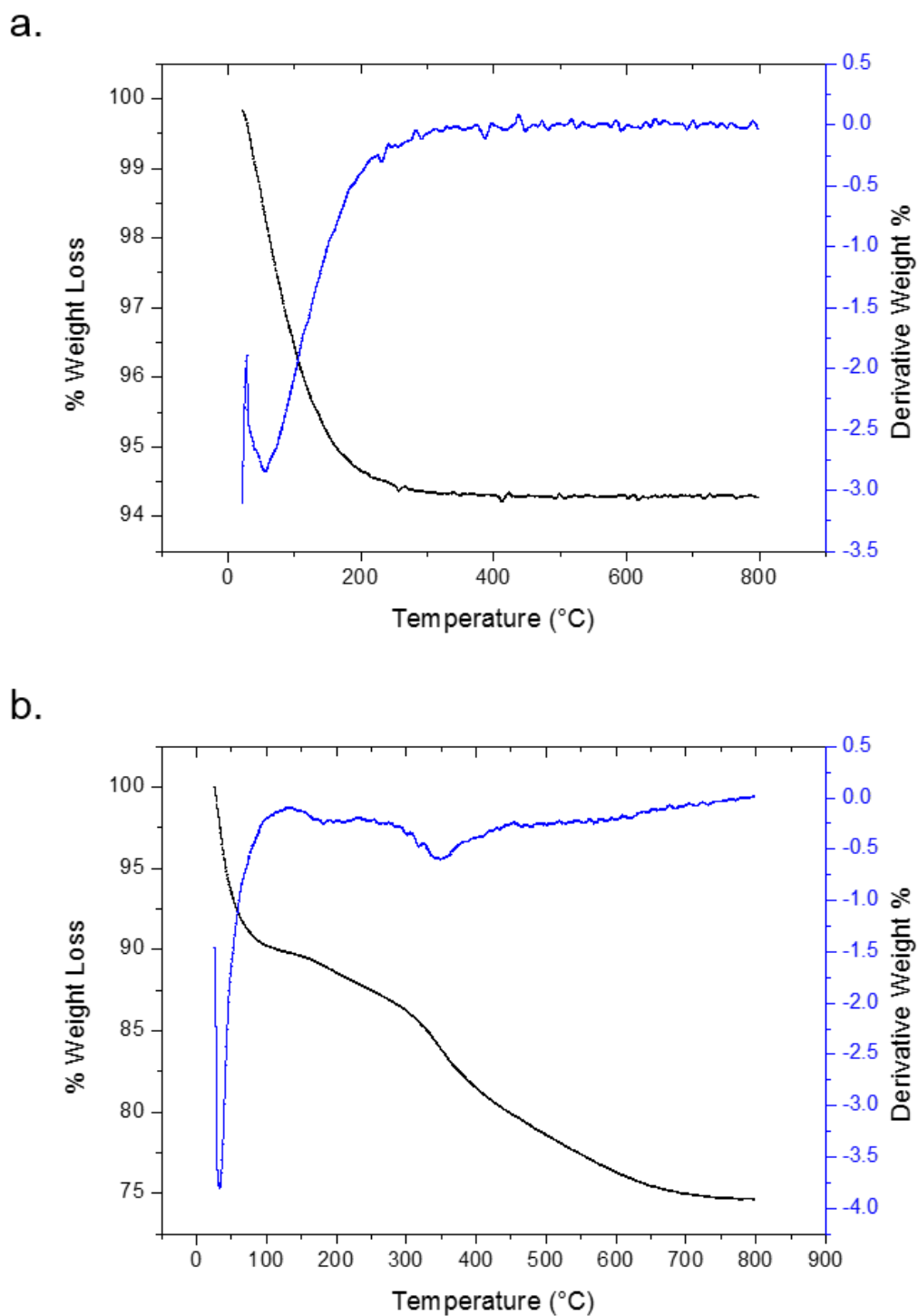


Figure 5.4 TGA analysis of SBA (a.) and SBA-APTES (b.).

The weight loss curves, measured using TGA analysis of SBA and SBA-APTES are shown in Figure 5.4. Regarding SBA, the initial weight loss of 4.8 % is associated with the removal of physisorbed water. The further weight loss of 0.8 % is due to the removal of chemisorbed water. The final weight loss 0.1 % was then attributed to the silanol groups which decompose to release water and subsequently the formation of silane bridges on the SBA surface. On the other hand, the TGA analysis of SBA-APTES reveals the presence of APTES on the SBA surface. The initial weight loss of 10.4 % was associated with the removal of physisorbed water, while a further 3.5 % was from the removal of chemisorbed water on the SBA-APTES surface. The decomposition of the APTES can be seen over the 300 - 400 °C range and resulted in an overall weight loss of 4.65 %. The final weight loss is associated with the dehydroxylation by condensation of silanols on the surface of the SBA-APTES (Maria Chong, et al., 2003). The larger weight loss observed with respect to SBA has been attributed to the presence of the amino groups. Those groups have high thermal stability (above 250 °C) suggesting that the SBA-15 silica sample has a stabilizing effect on the temperature of decomposition of the surface species (Hernández-Morales, et al., 2012).

These results indicate that APTES has functionalised to SBA-15 and has been widely reported in the literature (Lazghab, Saleh, & Guigon, 2010; Moritz & Łaniecki, 2012; Szegedi, Popova, Goshev, & Mihály, 2011). The mechanism of SBA-15 functionalisation with APTES has been reported to be through the condensation or hydrolysis of the terminal Si – OH from the SBA-15 with the alkoxy group on the ATPES, releasing H₂O and forming a stable covalent bond between SBA and APTES and is shown in Figure 5.1

(Luan, Fournier, Wooten, & Miser, 2005; Moritz, et al., 2012; Vejayakumaran, Rahman, Sipaut, Ismail, & Chee, 2008).

5.4.2 OEO Loading and Antimicrobial Activity of SBA & SBA-APTES

The C and N elemental analysis of SBA, SBA-ATPES, SBA-OEO and SBA-APTES-OEO are shown in Table 5.1. Results indicate that going from SBA to SBA-APTES an apparent increased in the amount of N and C occurs due to the grafting of the APTES. Furthermore, after OEO loading, a significant increase in the amount of C was observed due to the carbon from secondary metabolites that make up EO's. In addition, the weight of SBA-OEO and SBA-APTES-OEO before and after loading was taken and showed a significant increase in weight than unloaded SBA materials. These results indicate that OEO has been successfully adsorbed into the SBA voids.

Table 5.1 Elemental analysis of C and N before OEO loading (SBA-15 and SBA-APTES) and after OEO loading (SBA-OEO and SBA-APTES-OEO).

Element	SBA-15	SBA-APTES	SBA-OEO	SBA-APTES-OEO
<i>C</i>	Nil	7.84	29.63	28.78
<i>N</i>	nil	2.85	nil	1.71

FTIR analysis was also used to confirm successful loading of OEO (Figure 5.3 c. and d.). Results indicate that the pure OEO spectra shows sharp characteristic peaks at 2959 cm^{-1} (-CH stretching), 1589 cm^{-1} (N-H bending), 1458 cm^{-1} (-CH₂ bending), 1253 cm^{-1} (C-O-C stretching), 1117 cm^{-1} (C-O-C stretching) and 937 cm^{-1} (C-H bending) (Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013). When the spectra of SBA-OEO and SBA-APTES-OEO are compared to unloaded spectra (unloaded spectra previously outlined in

section 5.4.1), the presence of the characteristic peaks of OEO are found to appear at the same wavenumber as the pure OEO, therefore, indicating that pure OEO was loaded into the SBA-15 or SBA-APTES support but no modification or interaction between the OEO and SBA-15 occurred (Hosseini, et al., 2013).

Results from the MIC assay show that both SBA-OEO and SBA-APTES-OEO have good antimicrobial activity against both Gram-negative *E.coli* and *P. fluorescens*, and Gram-positive *S. aureus* and *B. cereus* (Figure 5.5). Moreover, results indicate that APTES grafting onto SBA had no effect on the antimicrobial activity of OEO compared to SBA-OEO against Gram-positive *S. aureus* and *B. cereus* bacteria; however, a difference with respect to the antimicrobial activity against Gram-negative *E. coli* and *P. fluorescens* was observed. It was found that the susceptibility of *E. coli* and *P. fluorescens* was effected by the functionalisation of SBA with APTES and may perhaps be due the ATPES changing the release profile of the secondary metabolites of OEO and therefore, effecting the interaction of OEO with the bacterial cell (Stanzione, et al., 2017) and subsequently, affects the mode of antimicrobial activity of OEO. The exact mechanism of OEO antimicrobial activity is not fully understood; however, has been reported to be from a synergistic action of the secondary metabolites such as p-cymene, thymol, and carvacol present in oregano EO's (Hyldgaard, et al., 2012). In particular, carvacrol can disintegrate outer membranes of Gram-negative bacteria while in Gram-positive bacteria the membrane permeability is altered allowing permeation cations like H^+ and K^+ (Hyldgaard, et al., 2012) and p-cymene, while not inherently antimicrobial, has a high affinity for bacterial cell membranes where it can substitute itself into the cell membrane altering the physiological barrier properties facilitating easier access for other more potent

antimicrobial compounds (Burt, 2004). Moreover, the shape of the bacteria has been also reported to affect the antimicrobial efficacy of EO's, where rod shaped cell have been reported to be more sensitive than coccoid shaped cells (Sullivan, et al., 2018). Moreover, results indicated that Gram-positive showed a greater susceptibility to than Gram-negative bacteria to SBA-OEO and SBA-APTES-OEO. Increased Gram-positive bacteria susceptibility has been widely reported in the literature and is believed to be through the lipophilic ends on lipoteichoic acid in the cell membrane of Gram-positive bacteria enabling the penetration of hydrophobic EO constituents into the internal cell structure (Tongnuanchan & Benjakul, 2014). Conversely, the reduced susceptibility of Gram-negative bacteria was attributed to the role of extrinsic membrane proteins and cell wall lipopolysaccharides limiting the diffusion of hydrophobic EO compounds into the microorganism (Tongnuanchan, et al., 2014).

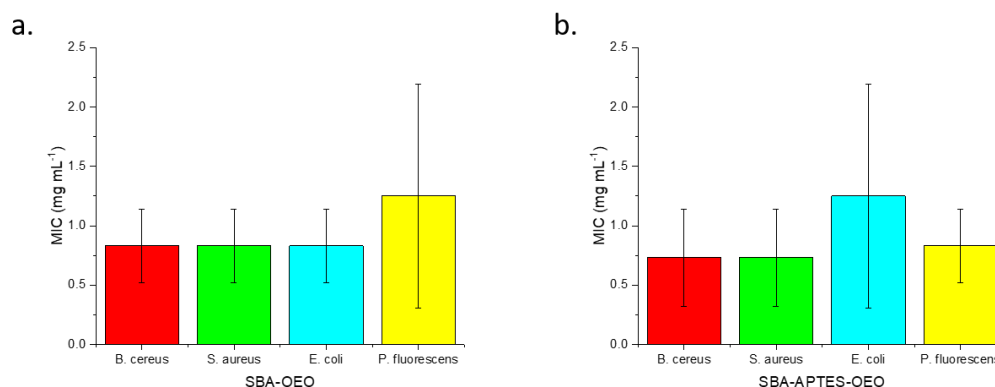


Figure 5.5 Minimum Inhibition Concentration of SBA-OEO (a.) and SBA-APTES-OEO (b.) against *B. cereus*, *S. aureus*, *E. coli* and *P. fluorescens*.

5.4.3 Attachment of GPTS to Si Coupons to develop Si-GPTS-ATPES-SBA

Materials

Figure 5.1 shows a schematic representation of the functionalisation of a surface with GPTS followed by reaction with SBA-APTES to form a Si-GPTS-APTES-SBA active surface and was characterised using a combination of SEM, XPS, AFM, ellipsometry, and contact angle measurements. Dynamic contact angle measurements of Si – OH (piranha treated Si), Si-GPTS and Si-GPTS-APTES-SBA are shown in Table 5.2. For piranha treated Si wafers (Si-OH), the wettability was found to be 8.57° ; however, after functionalisation of the Si-OH surface with GPTS, the wettability decreased to 56.90° due to the substitution of hydrophilic hydroxyl sites with the grafted GPTS chains which are more hydrophobic (Lazghab, et al., 2010). Regarding Si-GPTS-APTES-SBA, the wettability was found to decrease with respect to Si-GPTS to 26.23° . In addition, the dynamic contact angle of Si-GPTS and Si-GPTS-APTES-SBA was also measured using the dispersive solvent diiodomethane and results showed a contact angle of 39.90° and

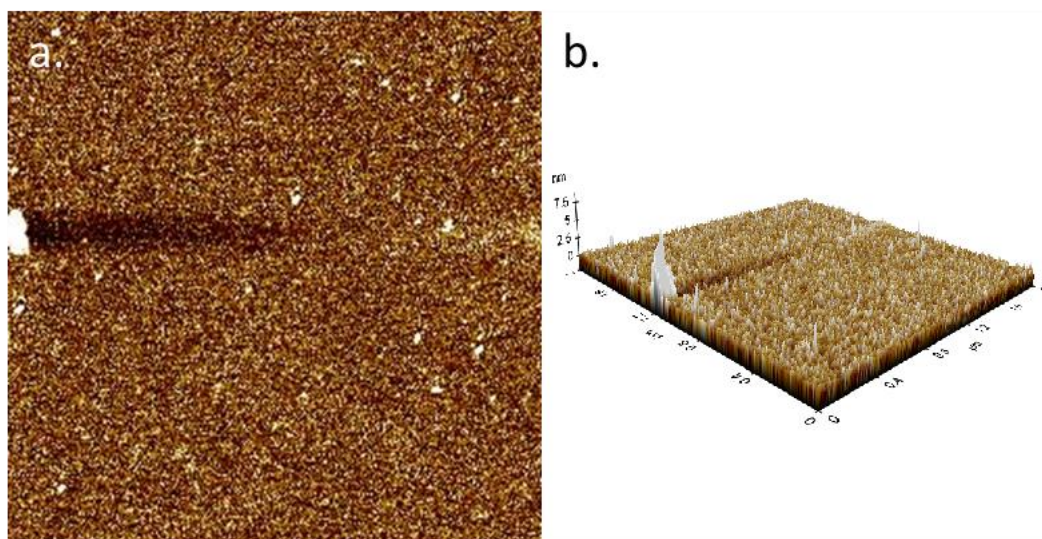


Figure 5.6 AFM images of Si-GPTS showing both 2D (a.) and 3D (b.) topographical images

26.23 ° for Si-GPTS and Si-GPTS-APTES-SBA, respectively. Moreover, from these results, the surface free energy was determined using the Owens-Wendt model (Eq. 1.). Results indicate that the surface free energy was 48.60 and 66.70 mJ m⁻² for Si-GPTS and Si-GPTS-APTES-SBA, respectively.

Additionally, the thickness and topographical feature of Si-GPTS were measured using ellipsometry (data not shown) and AFM analysis (Figure 5.6), respectively. Film thickness of the GPTS layer on a Si coupon was measured to be 18 nm, with a surface roughness of 1 nm. AFM analysis shows that Si-GPTS has a smooth topographical surface with small agglomerate features and may perhaps be due to excessive nucleation of GPTS to Si-OH surface and the surface roughness (Ra) was in agreement with spectroscopic ellipsometry data.

Table 5.2 Dynamic contact angle and surface free energy of Si-GPTS and Si-GPTS-APTES-SBA

Sample	Contact Angle	Contact Angle	Surface Free
	(H ₂ O) (°)	(I ₂ CH ₂) (°)	Energy (mJ m ⁻²)
<i>Si-GPTS</i>	56.90 ± 2.1	39.90 ± 0.5	48.6 ± 1.8
<i>Si-GPTS-APTES-SBA</i>	27.63 ± 3.3	26.23 ± 5.3	66.70 ± 5.8

Furthermore, XPS was used to examine the binding energies of Si 2p, O 1s, N 1s and C 1s core electrons of Si-GPTS-APTES-SBA (Figure 5.7). The XPS spectrum consists of peaks at 103 eV and 97.5 eV from organic and elemental silica, respectively. Moreover, the O 1s scan shows peaks at 532 and 529 eV which are attributed to Si-O₂ and organic C – O, respectively (Du, He, Liu, Ding, & Fang, 2011). The C 1s scan also revealed peaks

at 284 eV from C – H and C – C of the alkyl group of GPTS and also from adventitious hydrocarbon contamination, while the peak at 286.6 eV was from C – O – C and the oxirane ring of the GPTS, suggesting that the methoxy groups of GPTS have attach to the

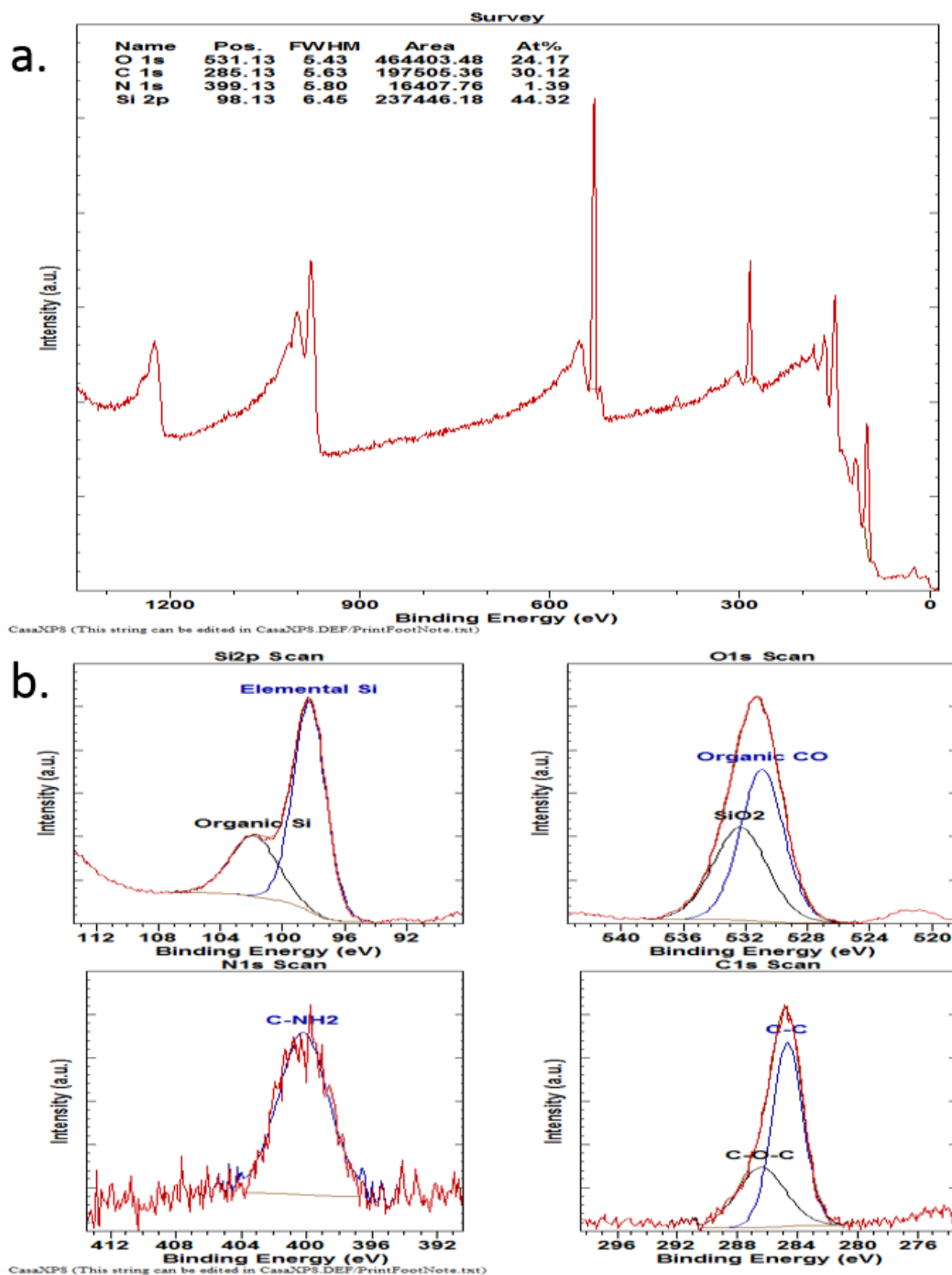


Figure 5.7 XPS survey of Si-GPTS-APTES-SBA

piranha treated Si substrate while the epoxide moieties orientate outward and are available for further reactions (Zengin, Tamer, & Caykara, 2015). In addition, peaks occurred in the N 1s spectra as a result of the amine groups from the APTES. These peaks in the N 1s spectra, were distinguished three different components related to: non-protonated, primary amino groups (399.6 eV), formamide groups (400.3 eV), and hydrogen bonded or protonated amino species (401.5 eV) (Wach, et al., 2017). Furthermore, from the XPS survey of and Si-GPTS-APTES-SBA, the percentage N was found to be 1.39 % and arises from the amino group of the APTES and therefore, it was determined that the attachment of SBA-APTES to the surface of Si-GPTS has occurred.

SEM analysis of Si-GPTS-APTES-SBA surfaces are shown in Figure 5.8. Results indicate that SBA-APTES was bound to the Si-GPTS surface which had been gently washed with deionised water to remove any unbound SBA-APTES from the Si-GPTS

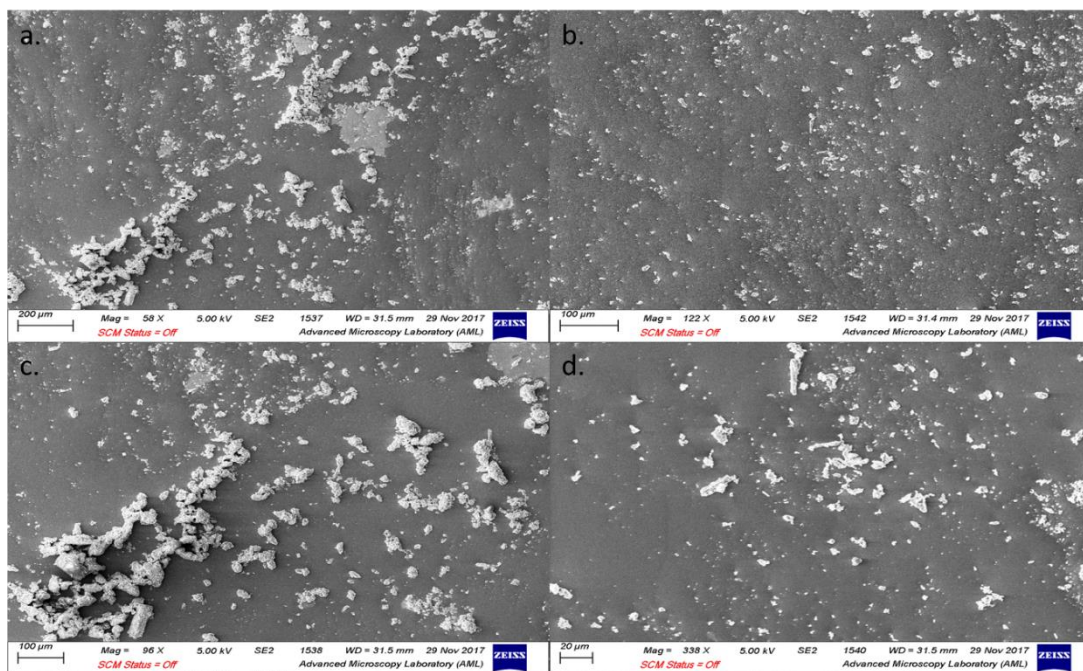


Figure 5.8 SEM of Si-GPTS-APTES-SBA surface at 200 (a.), 100 (b. – c.) and 20 (d.)

µm

surface. Moreover, SEM shows that the SBA-APTES is not forming an agglomerate multilayer, but isolated “specks”.

These results indicate that the SBA-APTES has been functionalised to the Si-GPTS modified surface (Figure 5.1). GPTS can attach to piranha treated Si wafers due to the treatment providing a high density of –OH moieties on the Si wafer surface and facilitates the functionalisation of the Si surface through silanisation with the ethoxysilane groups of GPTS via a hydrolysis reaction mechanism as outlined elsewhere (Vejayakumaran, et al., 2008; Wach, et al., 2017). The attachment of SBA-APTES to Si-GPTS is assumed to occur via a nucleophilic epoxide ring opening reaction between the amine groups from APTES and oxirane ring from GPTS resulting in the covalent grafting of SBA-APTES to Si-GPTS (Kamra, et al., 2015; Rajan, et al., 2018).

Nevertheless, when disk diffusion assay was carried no antimicrobial activity was observed (data not shown). This may perhaps be due to several factors such as (i) insufficient amount of SBA-APTES attached on the surface of the Si-GPTS, (ii) the slow release of OEO from SBA-APTES, or (iii) the interaction of APTES with the GPTS surface reducing the number of available pores for adsorption of the OEO, therefore, “blocking” its uptake into the mesospheric voids.

5.5 Conclusions

Herein we have presented an effective method to attach SBA-APTES to a GPTS modified surface and have demonstrated the use of SBA-OEO and SBA-APTES-OEO mesoporous material as an effective support material for OEO. The modification of SBA-15 with APTES was found to marginally improve the antimicrobial activity, compared to unmodified SBA, against common food spoilage microorganisms *E. coli*, *B. cereus*, *S.*

aureus, and *P. fluorescens*. However, when SBA-APTES was attached to a GPTS modified surface no antimicrobial activity was observed. Therefore, results from this study indicated that the attachment of SBA to a functionalised surface has significant roadblocks to its application in food packaging as insufficient SBA was attached to the GPTS modified surface and therefore, there was insufficient OEO on the surface, exacerbated by the lower antimicrobial effectiveness of OEO compared to other NAM's such as CS and CS NP. Consequently, the attachment of SBA to food packaging would appear not to be an effective or simple method to deliver antimicrobial OEO. However, given the strong antimicrobial activity and GRAS nature of SBA-OEO and SBA-APTES-OEO, they could potentially be applied via a simple "sprinkle" method (like salt) directly on a food product. Moreover, due to the strong absorption properties of SBA materials, other EO's could be used such as thyme and rosemary etc. for a suitable match of food and flavour. Additionally, Si-GPTS-APTES-SBA modified surfaces we have presented may find applications elsewhere such as biomedical devices, nutraceuticals etc.

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Chapter 6: Antimicrobial Effect of Benzoic and Sorbic Acid Salts and Nano-solubilisates against *Staphylococcus aureus*, *Pseudomonas fluorescens* and Chicken Microbiota Biofilms

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This Chapter was published as:

“Sullivan, D. J., Azlin-Hasim, S., Cruz-Romero, M., Cummins, E., Kerry, J. P., & Morris, M. A. (2020). Antimicrobial effect of benzoic and sorbic acid salts and nano-solubilisates against *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microbiota biofilms. *Food Control*, 107, 106786”

6.1 Abstract

The objective of this study was to evaluate the antimicrobial effects of benzoic and sorbic acid salt and their nano-solubilisates against planktonic and biofilm cultures of *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microbiota. The antimicrobial activity was affected by the particle size of the organic acid antimicrobials, the Gram-strain, and the type of culture (planktonic or biofilm) used. The organic acid nano-solubilisates were significantly ($P<0.05$) more effective compared to their organic acid salt counterpart with respect to both planktonic and biofilm cultures. However, biofilms of *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microbiota were significantly ($P<0.05$) more resistant to both organic acids salts and nano-solubilisates compared to the planktonic cultures. The physicochemical properties of the organic acids antimicrobials was assessed using particle size analysis, Fourier Transform Infrared (FTIR) spectroscopy and Atomic Force Microscopy (AFM) analyses. Biofilm formation after 24 h was quantified using a crystal violet assay and the minimum inhibition concentration against planktonic and biofilm cultures was also determined. The unique physiochemical properties of these nanomaterials may allow for potential applications in the development of naturally derived antimicrobial active packaging materials.

6.2 Introduction

Significant research has been carried out on the effects of naturally derived antimicrobials materials (NAM's) against planktonic bacteria; however, these only account for approximately 0.1 % of the global bacterial biomass (Bjarnsholt, Ciofu, Molin, Givskov, & Høiby, 2013). Under appropriate conditions, planktonic bacteria can multiply rapidly; however, their rapid proliferation makes planktonic bacteria more susceptible to pH change, fluctuations in temperature and the presence of

antimicrobials (Almasoud, Hettiarachchy, Rayaprolu, Horax, & Eswaranandam, 2015). Food processing and packaging environments can provide ideal conditions for the rapid growth of planktonic microorganism; however, modern cleaning techniques and practices have significantly reduced potential sources of contamination (Simões, Simões, & Vieira, 2010). While planktonic bacteria remain a source of contamination that can result in food spoilage, it has been reported that the majority of food spoilage from microorganisms arises from biofilms which are the source of up to 65 % of food contamination (Paraje, 2011). A biofilm is a community of microbes that collate together and attach on a surface and forms a self-produced, protective outer layer made from extracellular polymeric substances (EPS) (Donlan, 2002; Pande, McWhorter, & Chousalkar, 2018). Biofilms can be made up of either a homogeneous or heterogeneous combinations of Gram-positive and Gram-negative bacteria (Kumar & Anand, 1998) and can attach onto surfaces through either a passive mechanisms such as gravity, electrostatic forces or van der Waals interactions or through an active mechanism *via* the pili or flagella structures of these microorganisms. Typically, biofilms will preferentially attach to lipophilic surfaces such plastics, rubbers and polytetrafluoroethylene (PET) (Brooks & Flint, 2008; Frank, 2003; Simões, et al., 2010). Nonetheless, biofilms are also found to form on various materials used in food processing and packaging industry including; stainless steel or glass (Brooks & Flint, 2008; Chae, Schraft, Truelstrup Hansen, & Mackereth, 2006; Srey, Jahid, & Ha, 2013). Food packaging and processing environments provide suitable conditions for the formation of biofilm such as temperature, pH (Brooks & Flint, 2008), relative humidity (Lee, Bae, Lee, & Lee, 2015), and the availability of minerals and nutrients (Donlan, 2002). Upon the formation of a biofilm mass on a substrate, the gene expression of the collated bacteria changes to allow the growth of EPS which protects

the biofilm from environmental changes and stressors such as detergents or sanitisers including; hypochlorite's, quaternary ammonium compounds and antibiotics (Chae, et al., 2006; Lee, et al., 2015; O'Toole, 2001; Zottola, 1995). Biofilms can propagate through the release of bacterial EPS fragments that detach from the biofilm mass and can attach elsewhere to start the formation of new biofilm mass (Bridier, et al., 2015).

While many sanitising strategies such as clean-in-place have been successfully employed to control the growth of planktonic bacteria, biofilms are more resistant to these types of treatments due to their more complex structure and composition (Bridier, et al., 2015). Therefore, novel strategies are required to control biofilm proliferation and studies have shown that biofilm formation and contamination can be significantly reduced through the application of organic acids (Almasoud, et al., 2015; Amrutha, Sundar, & Shetty, 2017). Two organic acids widely used in the food industry as preservatives are sorbic and benzoic acid. These organic acids occur naturally in many fruits or plants and are also produced by the gut in millimolar quantities (Pande, et al., 2018). Moreover, their application in food products are favourable due to properties including; their Generally Recognized as Safe (GRAS) status from the FDA, clean label, colourless and tasteless properties and a historical use in food preservation (Berger & Berger, 2013; Sieber, Butikofer, & Bosset, 1995). Organic acids salts have been previously used on biofilms by Almasoud, et al., (2015) who applied lactic and malic acid solutions *via* electro-spraying onto spinach and cantaloupe rinds inoculated with biofilms of *E. coli* O157:H7 and *Salmonella* Typhimurium where it was found that the combined treatment of lactic acid and malic acid (2 + 2 % w/v) showed the greatest log reduction of 4.14 and 3.6 (CFU/disk) on spinach and cantaloupe rinds inoculated with biofilm cultures, respectively. Moreover, organic acid preservatives either individually or in combination have been applied on

a variety of food products such as buffalo meat (Malik & Sharma, 2014), beef steak microbiota (Clarke, et al., 2016), frankfurters (O'Neill, Cruz-Romero, Duffy, & Kerry, 2018), cooked turkey meat (Contini, et al., 2014), broccoli sprouts (Chen, et al., 2019), and lettuce (Zhao, Zhang, & Yang, 2017).

The recent development of engineered natural antimicrobial (NAM) nanomaterials has shown promising potential for the application of nanotechnology in the food sector (Azlin-Hasim, et al., 2015). These NAM materials overcome many of the toxicological and environmental concerns posed by use of metal derived nanoparticles (Gaillet & Rouanet, 2015), while maintaining their enhanced properties such as greater antimicrobial activity over their bulk counterparts (Dutta, Dey, Shome, & Das, 2011; Ngan, et al., 2014; Paomephan, et al., 2018; Pilon, et al., 2015). Studies have reported that biofilms are more susceptible to nanomaterials due to their smaller dimensions which facilitates greater accesses to the internal structures and organs of biofilms, disrupting internal organ functions (Epstein, Pokroy, Seminara, & Aizenberg, 2011). Organic acid salts and nano-solubilisates of sorbic acid and benzoic acid are currently commercially available and Cruz-Romero, Murphy, Morris, Cummins, & Kerry, (2013b) applied these materials against *P. fluorescens*, *S. aureus*, *Bacillus cereus*, *Escherichia coli*, chicken microbiota and cheese derived microbiota and found that nano-sized solubilisates of benzoic acid and sorbic acid had significantly ($P<0.05$) higher antimicrobial properties than their non-nano equivalents.

To the best of our knowledge there is little information regarding the antimicrobial effect of organic acids (sorbic and benzoic acid) and their commercially-available nano-sized solubilisate equivalents against planktonic and biofilm cultures of *Staphylococcus aureus* (*S. aureus*), *Pseudomonas fluorescens* (*P. fluorescens*) and chicken microbiota. Therefore, the aim of this study was evaluate and compare the

antimicrobial activity of sorbic and benzoic acid salt and their commercially available nano-sized solubilisates against planktonic and biofilm cultures of *S. aureus*, *P. fluorescens* and chicken microbiota.

6.3 Materials and Methods

6.3.1 Materials

Benzoic acid (BAS) and Sorbic acid (SAS) were purchased from Sigma-Aldrich (Ireland). Water- and fat-soluble 4 % sorbic acid solubilisate (SASB) and 12 % benzoic acid solubilisate (BASB) were obtained from Aquanova (Aquanova, Darmstadt, Germany). Crystal violet (CV) and glacial acetic acid were obtained from Fisher Scientific, Ireland. For all tests, Mueller-Hinton broth (MHB, Oxoid) was used as growth media and for any culture dilution. The bacterial loads were determined using spread plate method on plate count agar (PCA) (Merck, UK). Sterile distilled water was used for any washing process.

6.3.2 Physical Characterisation of Organic acid Salts and Nano-solubilisates

Particle size analysis of SASB, and BASB was carried out using a Malvern Zetasizer Nano Series HT (Malvern, U.K.). Solubilisate solutions were loaded into a disposable cell (ZEN0040) and analysis was performed at 25 °C using a scattering angle of 173°. The particle size distribution and polydispersity index (PDI) of the SASB and BASB solutions was determined using the Mark–Houwink method and values are average values of duplicate measurements with 3 replicates per sample. Fourier transform infrared spectroscopy (FTIR) analysis of SAS, BAS, SASB, and BASB was performed on a Varian 660-IR spectrometer (Varian Resolutions, Varian Inc, Victoria, Australia) using a diamond crystal ATR Golden Gate (Specac). Scans were taken with 32 scans at 2 cm⁻¹ resolution in a wavenumber range from 4000 - 500 cm⁻¹. For the Atomic Force Microscope (AFM, Park systems, XE-100, South Korea) measurement, silicon

wafer coupons (2 cm x 2 cm, PI-KEM Limited, Tamworth, UK) were ultrasonicated (Cole-Palmer 8891, IL, USA) for 30 min twice in absolute ethanol and then homogenised diluted solutions of 0.1 w/v % SASB and BASB solutions were spin coated (Specialty Coating Systems, 6800 spin coat series, IN, USA) onto the cleaned Si wafers at 3000 rpm for 30 s and dried under a stream of nitrogen (N₂) gas. AFM scans were performed in non-contact mode with high resolution, silicon micro-cantilever tips. Topographic images were recorded at a resonance frequency of 270-300 kHz.

6.3.3 Planktonic and Biofilm Bacteria Growth

The following planktonic and biofilm forming pure bacterial strains were used: *S. aureus* (NCIMB 13062) and *P. fluorescens* (NCIMB 9046). Before use, planktonic cultures of *S. aureus* and *P. fluorescens* were grown for 18 h at 30 °C (*P. fluorescens*) or 37 °C (*S. aureus*) in Mueller-Hinton broth (MHB) (Oxoid, UK) under constant agitation at 170 rpm on an orbital shaker (Innova 2300, New Brunswick™, Germany). A microbiota isolated from raw chicken breast fillets sourced locally was also used where chicken microbiota was isolated from raw chicken breast fillets *via* 10 g of chicken being taken aseptically and placed in a stomacher bag to which 90 ml of sterile MHB was added. The mixture was homogenised for 180 s in a stomacher (Colworth Stomacher 400, Seward Ltd., England) and 10 mL of the resulting homogenate was transferred into a sterile Sterilin™ tube with screw cap (Sterilin, UK) and incubated at 37 °C for 18 h under constant agitation as previously outlined.

6.3.4 Antimicrobial test on Planktonic Bacteria

The antimicrobial activity of the organic acids and nano-solubilisates against planktonic cultures was tested by determining the minimum inhibitory concentration (MIC) in a 96-well flat bottom plates (Sarstedt Inc., NC, USA) with alpha numeric

coordination system (columns 1-12 and rows A-H) according to the NCCLS broth microdilution method (Wayne, 2002). Briefly, rows A to F wells were filled with 100 μL of double strength MHB. A serial 10-fold dilution of an overnight grown microorganisms was carried out using MHB as diluent to obtain a final concentration of $\log 5 \text{ CFU ml}^{-1}$. Two hundred microliter of the diluted target microorganisms was added into the wells of row H, columns 1-11 while 100 μL of sterile MHB was added to column 12. In each well of row G, 150 μL of the antimicrobial substance solutions were added (2 % w/w). Using a 12 channel pipette, 50 μL of antimicrobial substance solutions was serially transferred from each well in row G into the corresponding wells in row F and the process repeated to row B. After mixing, 50 μL was removed from each well in row B and discarded. Positive (row A) and negative growth controls (column 12) were included in each assay plate. Finally, using a 12 channel pipette, 15 μL of the standardized inoculum were pipette from each well in row H to the corresponding wells in row A followed by rows B – G. The inoculated plates were incubated for 24 h at 30 °C (*P. fluorescens*) or 37 °C (*S. aureus* and chicken microbiota). The lowest concentration showing inhibition of growth was considered to be the MIC for the targeted microorganism. Each experiment was performed in quadruplicate and with three independently grown cultures. The initial bacterial load (Colony-forming units (CFU)/mL) of *P. fluorescens*, *S. aureus* and chicken microbiota was determined after the appropriate dilution was placed in duplicate on PCA plates and incubated for 24 h at 30 or 37 °C, respectively. After MIC determination, an aliquot of 0.1 mL from all wells without visible bacterial growth was plated onto plate count agar plates. The PCA plates were then incubated for 18 h at 30°C for *P. fluorescens* or 37°C for *S. aureus* and chicken microbiota. After incubation, the concentration of BAS, SAS, BASB or SASB at which there was no visible growth of

the bacteria on the PCA plates, was noted as the Minimum Bactericidal Concentration (MBC).

6.3.5 Biofilm Formation

The biofilm formation was carried out using a procedure outlined by Evaristo, et al., (2014) with some modifications. An overnight grown pure culture of bacteria or chicken microbiota isolated as outlined in section 2.3 was 10-fold serially diluted to log 6 CFU ml⁻¹ and 100 µL of the diluted target microorganism was added into each well of a 96-well flat bottom plate except in row H and column 12. Then, the 96-well plates were incubated for 24 h at 30 or 37 °C as previously outlined. The formation of biofilm was confirmed by the addition of 100 µL of sterile water without CV present to each well and sonicated for 8 min to remove unattached cells from the surface of the well. To determine the bacterial load of the biofilm, a serial dilution of the biofilm was carried out and enumerated using PCA as the growth medium.

6.3.6 The Quantification of Biofilm Formation

After the incubation process, the cultures in the microplates were removed by transferring the liquid into a large empty tray and shaken vigorously to remove the remaining excess liquid. Any unattached bacterial cells were removed by further washing the wells with sterile water from a wash bottle (Azlon Plastics, UK). The washing process was repeated three times and then the 24-well plates turned up-side down into a sterile absorbent paper and tapped vigorously on the sterile absorbent paper towels to remove any remaining water. The 24-well plates were turned upwards and left to dry in a laminar flow (Airclean 600 PCR Workstation STAR LAB) for 2 h. Then, 125 µL of 0.1 % (v/v) CV solution was added to each well and left for 15 min at room temperature (~ 20 °C). The wells were then washed three times using sterile distilled water as outlined above to remove any excess dye and then allowed to dry for

1 h in a laminar flow. In order to quantify the planktonic cells forming the biofilm, 125 μ L of 30 % acetic acid was added to each well to dissolve the CV and left for 15 min at room temperature. The solubilised CV solution was transferred into an ultra-micro cuvette and the optical density (OD) of the solutions measured at 550 nm in a UV-Vis spectrophotometry (UV Mini 1240, Shimadzu Instruments, Jiangsu, China) using 30 % acetic acid as the blank.

6.3.7 Antimicrobial Susceptibility of Biofilms to Antimicrobials

In order to determine the effect of the antimicrobial organic acid materials against biofilm formations of pure culture and chicken microbiota, suspensions were prepared as outlined in section 6.3.3. A 96-well plate containing biofilms were prepared using a procedure similar to that of formation and staining of the biofilms except no CV dye was added to the well plates. A serial dilution of antimicrobial substances were carried out in another set of sterile 96-well plates using the procedure outlined in the antimicrobial activity test, except no target microorganism were added in the row H. Then, 100 μ L diluted antimicrobial substance solutions in the sterile 96-well plates were transferred aseptically to another 96-well plate containing biofilms. The plates were then incubated for 24 h at 30 or 37°C as outlined above and the susceptibility of the biofilms to the antimicrobial monitored. The lowest concentration showing inhibition of growth was considered to be the MIC for the target biofilms.

6.3.8 Statistical Analysis

Statistical analysis was performed using the software STATGRAPHICS® centurion XV (Statpoint, Inc., USA). A difference between pairs of means was resolved by means of confidence intervals using Tukey's test. The level of significance was set at $P < 0.05$.

6.4 Results and Discussion

6.4.1 Physical Characterization of Organic Acid salts and Nano-solubilisates

The hydrodynamic particle size of BASB and SASB measured using a zetasizer was found to be 9 and 10.6 nm, respectively (Figure. 6.1). The polydispersity index (PDI), which is a dimensionless measure of the range of the particle size distribution calculated from the cumulant analysis, was recorded to be 0.247 for BASB and 0.212 for SASB.

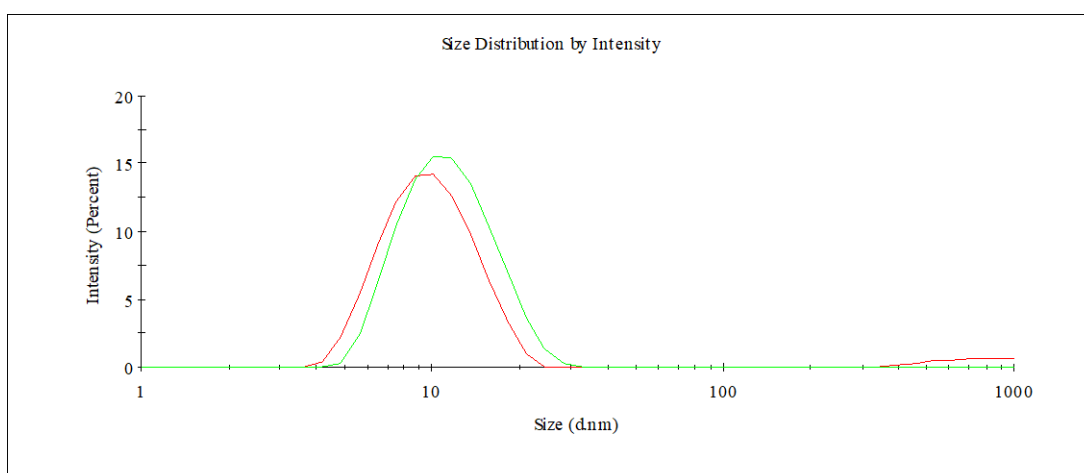


Figure 6.1 Particle size distribution (a) of benzoic acid nano-solubilise (—) and sorbic acid solubilise (—) measured using a Malvern Zetasizer Nano Series HT where organic acid solutions were loaded into a ZEN0040 disposable cell and analysis was performed at 25 °C using a scattering angle of 173°.

These PDI results indicate that the nano-solubilisates were monodisperse and had a narrow particle size distribution (Sullivan, Cruz-Romero, et al., 2018b). Due to the irregular and large size features of BAS and SAS, topographical imaging was carried out only on the nano-solubilisates. The morphological and topographical features of BASB and SASB measured using the AFM are shown in Figure. 6.2. Both BASB (Figure 6.2.a) and SASB (Figure 6.2.c) had a monodisperse and regular spherical morphology which are features typical of monodisperse micellar emulsion. Moreover,

AFM analysis indicated that the average particle diameter of BASB and SASB was 49.5 and 63.85 nm, respectively. In comparison, the particle size diameter of the BASB and SASB obtained using AFM analysis were up to 5.5 and 6 times larger than the particle size results obtained with the zetasizer which was a hydrodynamic light scattering measurement. The larger observed particle size diameter from AFM analysis may perhaps be due to a “pancaking” effect as when removed from aqueous solution, the morphology adopts a disc like shape due to the effects of gravity acting on the micelle. The FTIR spectra of BAS, SAS and commercially available nano-sized BASB and

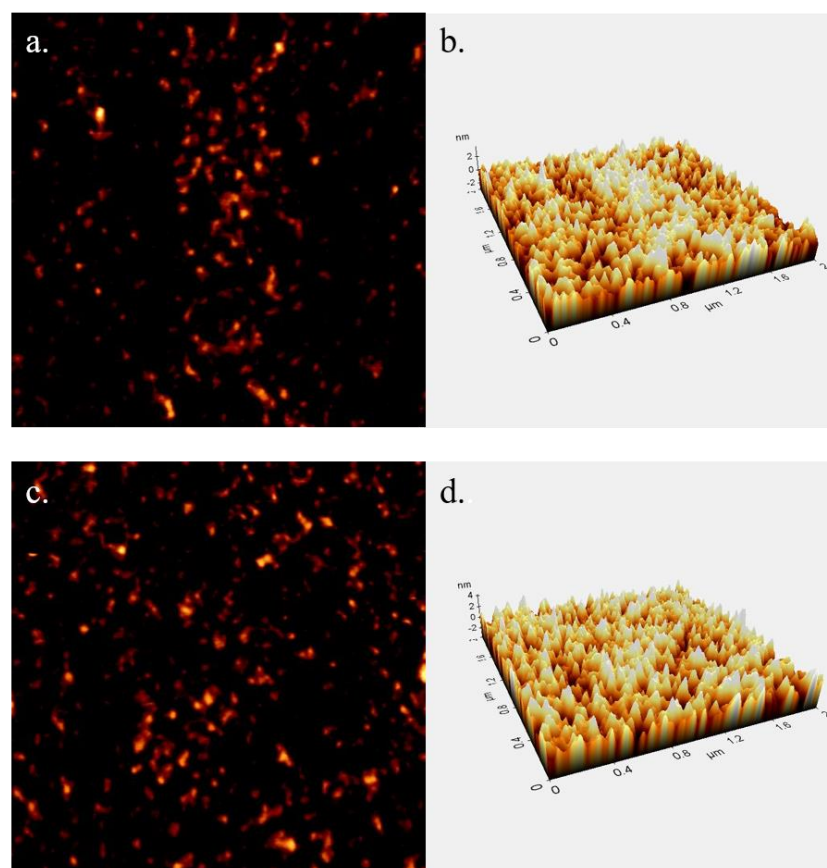


Figure 6.2 AFM topographical ($2 \times 2 \mu\text{m}$) (a.) and 3 D (b.) images of benzoic acid nano-solubilisate and AFM topographical (c.) and 3 D (d.) images of sorbic acid nano-solubilisate.

SASB are shown in Figure 3 a & b, respectively. While both BAS and SAS have similar functional groups their backbone structure is different as sorbic acid is a 6 carbon aliphatic chain monomer with a carboxylic acid group, whereas BAS has a 6 carbon phenol ring with a carboxylic acid attached. Nevertheless, the FTIR spectra of SAS and BAS showed different characteristic peaks. The FTIR spectra of SAS showed peaks at 2500 – 3500 cm^{-1} due to the O-H of the COOH stretching, 1694 cm^{-1} corresponding to C = O of acid stretching, C = C stretching for alkene at 1613-1638 cm^{-1} , bending CH_3 at 1377 cm^{-1} , stretching COH acid at 1266 cm^{-1} and at 998 cm^{-1} an out of plane for trans-alkene (El-Nemr & Mohamed, 2017). The FTIR spectrum of BAS showed characteristic peaks at 1650 and 2500 - 3500 cm^{-1} which are due to C = C and O – H stretches, respectively. The spectra of the nano-solubilisates showed peaks at 1100, 2900 and 3500 cm^{-1} which are associated with the emulsifying agent. With regards to the overlap of BAS and BASB, a peak can be seen at 715 cm^{-1} which was attributed to BAS; however, none of the SAS peaks are apparent in the SASB spectra.

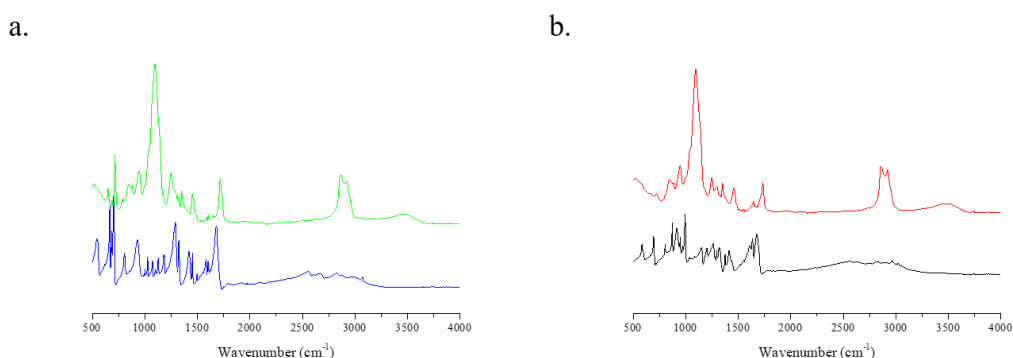


Figure 6.3 FTIR spectra of a. Sorbic acid (—) and sorbic acid nano solubilisate (—) and b. benzoic acid salt (—) and benzoic acid solubilisate (—).

6.4.2 Quantification of Biofilm Formation using Crystal Violet Assay

The formation of biofilms was quantified using a crystal violet assay (Figure 6.4.). For the crystal violet assay, the OD of *S. aureus*, *P. fluorescens*, and chicken microbiota biofilms formed on a 24-well plates were taken after 24 h incubation and the OD values were recorded to be 1.760, 0.643 and 0.406, respectively (Figure 6.4). Several factors have been reported to effect the growth of biofilms including the material of the microplate well plates which may influence the biofilm growth depending on the affinity of the biofilm forming microbes for either hydrophilic or hydrophobic surfaces (Naves, et al., 2008). Typically, microplates are made from polypropylene, a hydrophobic material that allows favorable surface attachment of biofilms through

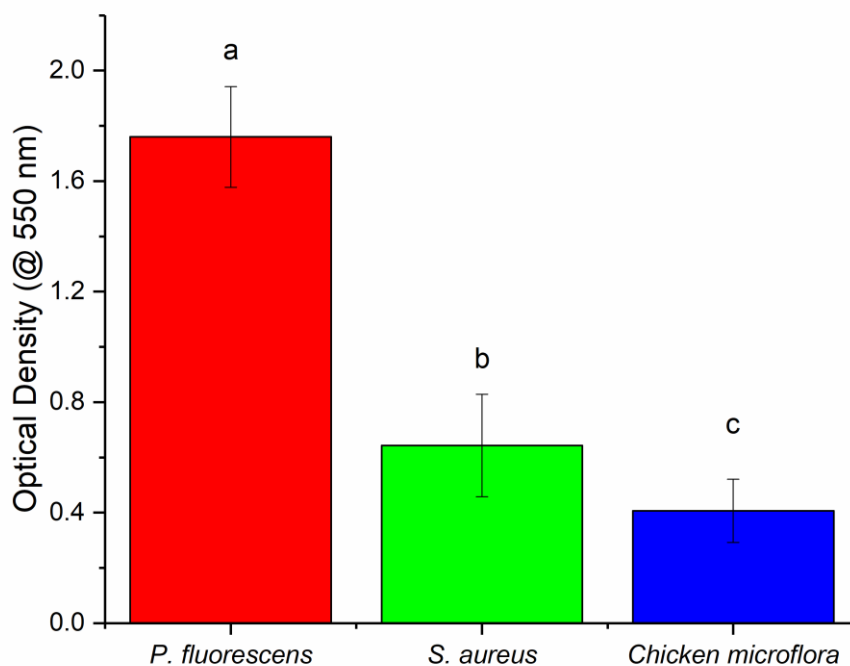


Figure 6.4 Quantification of biofilm cultures of *P. fluorescens* (■), *S. aureus* (■), and chicken microbiota (■) using optical density assay on biofilms grown in 24 well-plates. ^{a, b, c} Mean values with different superscripts indicate difference between values are significantly different ($P < 0.05$).

hydrophobic interactions while electrostatic interaction are predominantly seen in hydrophilic surfaces such as stainless steel (Di Ciccio, et al., 2015; Dutta, et al., 2011). Furthermore, authors such as Peeters, Nelis, & Coenye, (2008) and Stepanovic et al., (2000) have reported that the iodine dye used in crystal violet staining assay can also stain inactive cells and this may increase the observed OD.

6.4.3 Antimicrobial Activity of Organic Acids and Nano-solubilisates

The antimicrobial activity of BAS, SAS, BASB and SASB against planktonic and biofilms cultures of *S. aureus*, *P. fluorescens* and chicken microbiota were assessed using a minimum inhibition concentration (MIC) assay (Figure. 6.5 a & b). Particle size and the type of bacteria (Gram-positive or Gram-negative) affected significantly ($P<0.05$) the antimicrobial activity of BAS, SAS, BASB and SASB against planktonic and biofilms cultures. As expected, planktonic cultures of *S. aureus*, *P. fluorescens*, and chicken microbiota were more susceptible to BAS, SAS, BASB and SASB than their respective biofilms. Of the planktonic cultures, chicken microbiota was the least susceptible to the tested antimicrobial agents of which SASB was the most effective antimicrobial while SAS the least effective antimicrobial. The most susceptible culture was *S. aureus* where SASB was the most effective antimicrobial while the least effective was BASB. Furthermore, the MBC assay showed a higher antimicrobial efficacy of nano-solubilisates compared to their respective salt (Figure 6.5.c). Against *S. aureus*, it was observed that 1.27 and 2.3 times more BAS and SAS was required to have the same bactericidal effect compared to their respective nano-solubilisates. Similarly, 5 and 3.3 times more salt was required than nano-solubilisates against *P. fluorescens* and 4.2 and 3.07 times more salt than their respective nano-solubilisates was required against chicken microbiota.

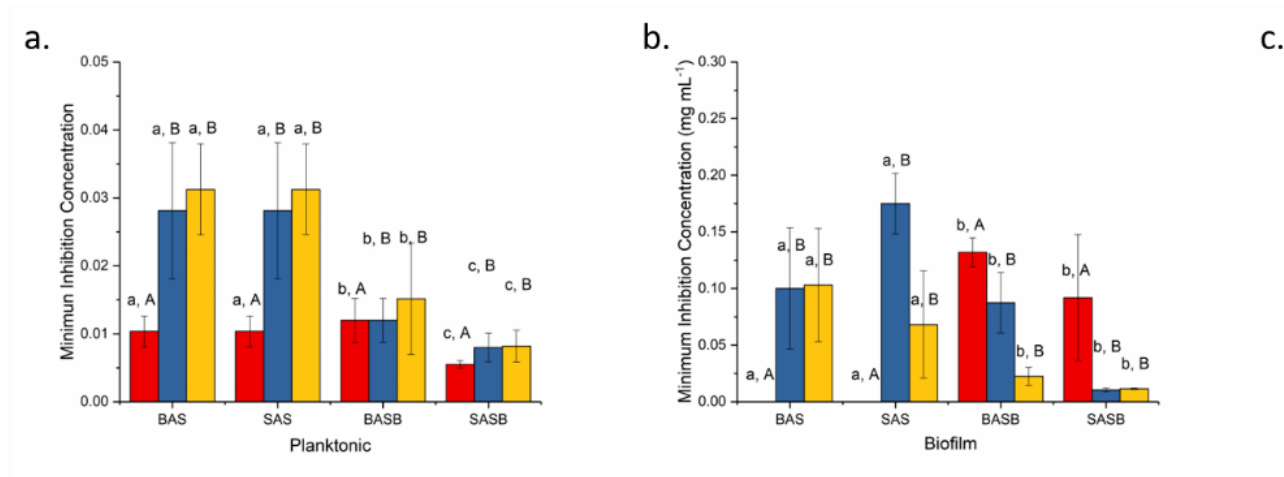


Figure 6.5 MIC of benzoic acid salt, sorbic acid salt, benzoic acid nano-solubiliser and sorbic acid nano-solubiliser against planktonic (a.) and biofilms (b.) cultures of *S. aureus* (■), *P. fluorescens* (■) and chicken microbiota (■) while in (c.) the MBC is shown. ^{a, b, c} Mean values with different superscripts indicate difference between BAS, SAS, BASB, and SASB are significantly different ($P < 0.05$). ^{A, B, C} Mean values with different superscripts indicate difference between *S. aureus*, *P. fluorescens* and chicken microbiota are significantly different ($P < 0.05$).

In comparison of MBC to their MIC values, it was observed that between 1.5 to 2.5 times more antimicrobials were needed to have a bactericidal effect. Interestingly, when comparing the antimicrobial effect of nano-solubilisates and salts against the Gram-type of pure cultures using an MIC and MBC assays it was observed that Gram-negative *P. fluorescens* was less susceptible compared to Gram-positive *S. aureus* in both instances, suggesting that organic acids are more effective against Gram-positive than Gram-negative planktonic bacteria. These results are in disagreement with Amrutha, et al., (2017) who reported that organic acids are more effective against Gram-negative microorganisms. This may be as a result of the proposed mode of antimicrobial action of organic acids which through their ability to dissociate into weak acids which then can cross bacterial membranes as a result of the equilibrium between their ionised and non-ionised forms, the latter of which can freely diffuse cross hydrophobic membranes initiating a collapse in the proton gradients that are necessary for ATP synthesis, as free anions will combine with periplasmic protons pumped out by the electron transport chain, and carry them back across the membrane without passage through the F1Fo ATP synthase (Amrutha, et al., 2017; Halstead, et al., 2015; Sullivan, Azlin-Hasim, et al., 2018a). Furthermore, as the pH of the organic acids environment decreases towards the pKa of the acid, its' antimicrobial effectiveness increases, as decreasing pH allows more protonation of the acid (and is in a pH dependant equilibrium). This reduces the polarity of the acid allowing its rapid diffusion through the cell membrane whereupon entering the alkaline internal pH of the cell, the internalised acid dissociates, increasing the concentration of protons and ions in the microbe, acidifying the cytoplasm, which in turn can cause acid-induced protein unfolding, membrane and DNA damage affecting cellular

functions such as glycolysis, cell signalling and active transport (Halstead, et al., 2015; Mani-López, García, & López-Malo, 2012). However, some studies have suggested that sorbic acid does not release enough protons for this type of inhibition and therefore, it is plausible that its hydrophobicity can interact with the cell membrane affecting its permeability resulting in intracellular leakage (Mani-López, et al., 2012). Studies have also reported that the chemical structure of the organic used will also have an effect on the antimicrobial activity (Akbas & Cag, 2016). In addition, it has been proposed that another mechanism is through the ability of organic acids to act as an oxidant by producing hydroxyl free radicals which can interfere with the functionality of components of the cell such as; lipids, proteins and DNA (Zhang & Yang, 2017). Moreover, chelation of macronutrient metals by organic acids has also been shown to reduce the ability of the bacteria to thrive (Mani-López, et al., 2012). While the mechanisms of bacterial inhibition was not carried out in this study; however, emerging techniques such as: nuclear magnetic resonance (NMR) spectroscopy or gas chromatography-mass spectrometry (GC-MS) have been successfully used to identify the induced stress effects caused by antimicrobials (Liu, et al., 2018; Liu, et al., 2017)

Factors such as Gram-strain or organic acid molecular weight (MW) have also been reported to affect the antimicrobial activity of organic acids with Gram-negative bacteria less susceptible than Gram-positive bacteria, potentially due the more complex cell membrane structure (Clarke, et al., 2016) and lower MW organic acids predominantly antimicrobial through the “weak acid” preservative theory (Mani-López, et al., 2012). Conversely, larger MW organic acids interact with bacterial cell walls through lipophilic interactions with the cell membrane as they are too large to rapidly pass through the cell

membrane (Mani-López, et al., 2012). Moreover, apparently the physical properties of the organic acids were found to affect the antimicrobial properties. When comparing the antimicrobial properties of the organic salts and nano-solubilisates, it was observed that nano-solubilisates were more effective against both planktonic and biofilm cultures of *S. aureus*, *P. fluorescens* and chicken microbiota. The greater observed antimicrobial activity of nano-solubilisates may be attributed to its physical properties such as smaller particle size (nano vs non-nano) and the greater surface area of nano-solubilisates compared to their bulk salt materials as reported by Yu, Ang, Yang, Zheng, & Zhang, (2017) and Cruz-Romero, et al., (2013a).

With respect to the antimicrobial activity against biofilms, BAS and SAS had no antimicrobial activity against biofilms of *S. aureus* due to the concentrations required to inhibit their growth being over their 3400 and 1560 mg L⁻¹ solubility parameters in water, respectively. Furthermore, BASB and SASB were found to be significantly ($P<0.05$) less effective against *S. aureus* biofilms compared to biofilms of *P. fluorescens* and chicken microbiota. This may be due to the more complex structure and composition of biofilms, as outlined previously. Unexpectedly, chicken microbiota biofilms were the most susceptible to the organic acid salts and nano-solubilisates. This may be due to the chicken microbiota being a complex heterogeneous mix of different Gram-positive and Gram-negative bacteria which compete with each other for nutrients and therefore, inhibit optimal growth whereas pure isolates of bacteria have no competition and will form a homogenous biofilm (Pande, et al., 2018).

When the susceptibility of planktonic and biofilms cultures to organic acid antimicrobials was compared, it was observed that for SASB up to 15.3, 1.37 and 1.5 times more

antimicrobial material was required against biofilms of *S. aureus*, *P. fluorescens* and chicken microbiota, respectively. For BASB, the MIC was 11 and 1.64 times higher against *S. aureus* and chicken microbiota biofilms compared to their planktonic counterpart; however, 0.2 times less BASB was needed against biofilms than pure cultures of *P. fluorescens*. Regarding organic acid salts, for BAS 5.26 and 7.29 times more was necessary against *P. fluorescens* and chicken microbiota and for SAS 9.21 and 7.29 more was necessary against *P. fluorescens* and chicken microbiota. These results are in agreement with Surdeau, Laurent-Maquin, Bouthors, & Gellé, (2006) who used the disinfectant Oxsil® 320N on planktonic and biofilm cultures of *S. aureus* and *Pseudomonas aeruginosa* and also observed that biofilms were less susceptible than planktonic bacteria. Moreover, it has been reported that biofilms can be up to a thousand times more resistant to antimicrobials than planktonic cells of the same strain (Akbas & Cag, 2016). This may in part be due to the ability of biofilms to form protective EPS layer and in addition their slow rate of growth and metabolism will reduce the absorption of organic acid antimicrobials (Djordjevic, Wiedmann, & McLandsborough, 2002). A proposed mode of antimicrobial activity of nano-solubilisates against biofilms is thorough amphiphilic interactions, facilitating the diffusion of nano-solubilisates into the biofilm matrix and resulting in individual cell membranes death (Dutta, et al., 2011).

6.5 Conclusion

Herein we have investigated the antimicrobial effect of organic acid salts and nano-solubilisates on planktonic and biofilm cultures of the food spoilage microorganisms *S. aureus*, *P. fluorescens* and on the microbiota isolated from locally sourced chicken fillets. Results from this study indicate that the assessed organic acid salts and nano-solubilisates

showed good antimicrobial activity against planktonic cultures; however, biofilms were found to be significantly more resistant. Moreover, it was found that nano-sized solubilisates of benzoic acid and sorbic acid had significantly ($P<0.05$) higher antimicrobial properties compared to their non-nano equivalents of which SASB had greatest antimicrobial activity. Overall, the findings of this study indicated that the unique physiochemical properties of organic acid nano-solubilisates show promising potential as an emerging clean label antimicrobial for use in the development of active and smart packing materials to preserve the quality of food products.

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Chapter 7: General discussion, relevance, future work, and conclusions

7.1 General Discussion

Research into natural antimicrobials (NAMs) as an additional hurdle (to product spoilage) in food packaging has significantly increased in recent years due to consumer demands for fresh-like, sustainable, minimally processed, and clean labelled food products. However, removing chemical preservative and additives from food products significantly reduces their shelf life. To overcome this, the use of NAM's such as chitosan, oregano and organic acid antimicrobial nanomaterials are becoming leading contenders to replace chemical preservatives due to their wide availability from renewable sources, relatively low cost, and ease of production. Furthermore, these materials are: biocompatible (in contrast to metal ion nanomaterials), are biodegradable, and have good antimicrobial activity against a wide range of food spoilage microorganisms.

This thesis provides a state-of-the-art study in NAMs. In Chapter 1, a literature review of commonly used NAM materials and their application in antimicrobial active packaging (AAP) systems was conducted. From this study, the prime candidates for the development of NAM nanomaterials and potential incorporation strategies into food products were identified. In Chapter 2 we examined the effect of parameters such as MW, pH, CS and TPP concentration and CS:TPP mass ratio on the formation of monodisperse CS NPs using either bottom-up self-assembly or top-down techniques. The results from this study showed that these factors significantly affected the particles size and monodispersity of CS NPs where optimal formation parameters for 90 nm NPs were found to be 0.1 % w/v L. MW CS at pH 4.6 and 3:1 (CS:TPP) mass ratio. When higher initial concentrations of CS was used, an external (top-down) force such as tip sonication was required to induce

monodispersity and reduce the particle size; however, the particle size reductions was > 300 nm. The antimicrobial activity assessment indicated that no significant ($P<0.05$) differences between native CS and CS NPs, compared on their weight per weight basis, was observed against common food spoilage microorganisms *E. coli*, *B. cereus*, *S. aureus*, and *P. fluorescens*. Nonetheless the unique physiochemical properties of CS NPs such as their monodispersity and small particle size suggest that their application in food packaging would be more favourable. To this end in Chapter 3, we have evaluated the effects of the aerosolisation of antimicrobial solutions of native CS and monodisperse CS NPs developed in chapter 2 in conjunction with a commercially available nano-solubilisate (CASB) as a means to deliver an antimicrobial coating to extend the shelf life of VSP hake fillets. Throughout storage, the microbiological (TVC, anaerobes, psychrotrophs, *Pseudomonas* spp., H₂S-producing bacteria, LAB, *E. coli*, and coliforms) and physicochemical (proximal composition, colour, pH, and lipid oxidation) properties of the hake fillets were assessed using recommended microbiological limits of acceptability set with reference to TVC. Aerosolisation treatments of antimicrobial solutions of CS, CS NP and CASB extend significantly the shelf life of VSP hake fillets by 40, 50 and 55 %, respectively compared to untreated control samples. The pH and colour physicochemical characteristics of hake fillets increased significantly ($P<0.05$) throughout storage; however, the TBARS values decreased significantly ($P<0.05$) and subsequently were below the acceptability limits. In addition, the proximate composition of hake fillets were unaffected by aerosolisation of NAM antimicrobial solutions. These results highlighted the potential of aerosolisation of antimicrobial solutions of NAM nanomaterials as part of the hurdle strategy for extending the safety and shelf-life of hake

fillets and to the best of our knowledge, is novel and has not been reported in the literature elsewhere.

Chapter 4 assessed the effect of surfactant type (Tween 20® or Pluronic 127™), surfactant-to-oil ratio (SOR), and treatments (untreated, IKA or tip sonication) on the monodispersity and droplet size of oregano nanoemulsions, whereafter, the most appropriate nanoemulsion was used for aerosolisation treatment onto hake fillets, as part of a hurdle strategy to enhance its shelf life. The smallest droplet size of 40 nm was observed using a P127 nanoemulsions with a 1:2 surfactant-to-oil ratio; however, greater antimicrobial activity was observed using Tween® 20 nanoemulsions in a surfactant to oil ratio of 1:1 and had an average droplet diameter 131 nm. Therefore, these nanoemulsions were chosen for aerosolisation due to their good antimicrobial activity small droplet size, and good monodispersity. Aerosolisation treatments of oregano nanoemulsions solutions extend significantly the shelf life of VSP hake fillets by 50 % compared to untreated control samples. Moreover, the pH and colour physicochemical characteristics of hake fillets increased significantly ($P<0.05$) throughout storage; however, the TBARS values decreased significantly ($P<0.05$) and subsequently were below the acceptability limits. In addition, the proximate composition of hake fillets were unaffected by aerosolisation of OEO nanoemulsion solutions. These results further confirms that the use of aerosolisation treatments as part of a hurdle technology to enhance the microbiological safety and shelf-life in raw hake fillets. The research carried out in this chapter demonstrates the potential use of this technology for shelf life and safety enhancement using an essential oil based nanoemulsions, expanding the knowledge in the field.

In chapter 5 the objective was to assess the antimicrobial loading ability of SBA-APTES with OEO and to covalently attach the APTES functionalised SBA to GPTS modified surface as a support material for OEO. To achieve this, SBA surfaces were initially functionalised with APTES to give an amine terminal groups on the SBA. Concurrently, Si wafers were piranha treated and functionalised with GPTS, to give an epoxy functionalised surface. The SBA-APTES was then bound to the surface through a suspected ring opening reaction to covalently bind the SBA to the surface. These materials and both SBA and SBA-APTES were loaded with antimicrobial oregano essential and their antimicrobial activity was assessed. Functionalisation of SBA with APTES was found to marginally improve the antimicrobial activity, compared to unmodified SBA, against common food spoilage microorganisms *E. coli*, *B. cereus*, *S. aureus*, and *P. fluorescens*. However, when SBA-APTES was attached to a GPTS modified surface no antimicrobial activity was observed, as a result of insufficient SBA support coverage.

In chapter 6 we have evaluated and compared the antimicrobial activity of sorbic and benzoic acid salt and their commercially available nano-sized solubilisates against planktonic and biofilm cultures of *S. aureus*, *P. fluorescens* and the microflora isolated from locally sourced chicken fillets. Results indicate that both organic acid salts and nano-solubilisates showed good antimicrobial activity against planktonic cultures; however, more antimicrobial resistance was observed against biofilms due to their more complex structure. Nevertheless, when comparing organic acid salts and nano-solubilisates to each other, the nano-solubilisate were the significantly ($P < 0.05$) more effective as an antimicrobial agent against both planktonic and biofilm cultures.

7.2 Scientific and Industrial relevance

The output of this thesis has both scientific and industrial relevance. Regarding the scientific relevance, this research has added knowledge to the field. For instance, we have developed CS NPs with a particle size of 90 nm through bottom-up self-assembly and showed the interactive effect of CS molecular weight and pH on the particle size and monodispersity of CS NPs. Furthermore, contrary to reports by authors such as Qi, Xu, Jiang, Hu, & Zou, (2004) and Ngan, et al. (2014), who found that antimicrobial activity of CS NP was greater than native CS, we observed that native CS and CS NPs have a similar antimicrobial activity. Moreover, we have demonstrated that the aerosolisation of NAM nanomaterial solutions (such as CS NPs) are an effective hurdle in enhancing the shelf life and improving the safety of food products. Moreover, to the best of our knowledge, the findings of this study have not been reported elsewhere. This thesis also reported on a method to graft an amine functionalised SBA support material to a GPTS modified surface for essential oils is possible. Lastly we assessed the antimicrobial effectiveness of organic acid salts and nano solubilisates against planktonic and biofilm cultures of *S. aureus*, *P. fluorescens* and the microflora isolated from locally sourced chicken fillets, showing that nano-solubilisates had greater antimicrobial effect and could be used to improve food safety.

Regarding the industrial relevance of this work, the focus on NAMs would perhaps make their use in food products more acceptable to the consumer. The physicochemical properties of these materials such as their biocompatible and biodegradability make them ideal candidates for use in food packaging and products. Moreover, the nanomaterials outlined herein can be synthesised with relative ease from cheap and naturally abundant

material such as: plants and shrimp shells. In particular self-assembly of CS NPs would be a favourable route due to their low cost nature and relative little impact on food, as highlighted in Chapter 3. However, the application of oregano nanoemulsions will require a more nuanced approach due to their strong impact on organoleptic properties, nonetheless, they also can be used as a multifunctional additive (i.e. as both an antimicrobial, antioxidant, and as a flavour enhancement).

One of the key findings of this thesis is the effectiveness of aerosolisation of antimicrobial NAM nanomaterials as a method of treatment to enhance shelf-life (Chapter 3 and 4). From an industrial perspective this technology may be suitable due to the low volumes of antimicrobial solution required, relative ease of application, and good coating coverage. Furthermore, this technology could be used on a wide variety of delicate, perishable, and high-value products such as fruits, raw meats and vegetables. Another industrially relevant finding from this thesis was the greater antimicrobial efficacy of organic acid nano-solubilisates over their counterpart salts and against planktonic and biofilm cultures of *S. aureus*, *P. fluorescens* and the microflora isolated from locally sourced chicken fillets.

It is also important to stress that the methods outlined here could form one part of strategy to significantly extend shelf-life. The NAMs formed an effective method for mitigating spoilage at the product surface (since they are applied as a fine spray coating). Moreover, their use does not have any implications in terms of regulation and should be a consumer friendly mitigation methods. Antimicrobial packaging could be used with perhaps strong antimicrobials (such as silver, however, affords problems in terms of regulation) at the exterior surface of the package to prevent ingress of microbial contamination. Coupled to

the use of antimicrobial soakage pads and other strategies. Combinatorial methods may afford shelf-life doubling or greater and this would have very significant impact on spoilage and waste in the food industry; a significant issue with the Food & Agriculture Organisation (FAO) estimating that one-third or over 1.3 billion metric tons of all edible food produced for human consumption is either lost or wasted annually throughout the supply chain.

7.3 Future Work

This thesis lays the ground work for the application of NAM materials through a variety of different strategies. For instance, further investigation into the aerosolisation applications of NAM nanomaterial solutions are needed. The work carried out in this thesis was a preliminary investigation in to the use of this technology with respect to food packaging application. As such, there is scope to investigate the effect of altering parameters such as aerosolisation rate, solution volume, etc, to ascertain their effects on the coating layer. Moreover the investigation of effects of the viscosity on aerosolisation would need to be carried to determine more precisely what properties a “good” aerosolisable solution necessitates. In addition, these solutions could also be multi-component solutions that either contain two different antimicrobials or could contain both an antimicrobial and an antioxidant that would be aerosolised concomitantly.

In another approach, these NAMs could be attached to the surface of packaging materials by direct incorporation into the packaging material via extrusion, surface coating, or through the development of surface architecture to “contain” and release the antimicrobial material over time. The latter may perhaps be achieved using a block-co-polymer such as polystyrene-block-polyethylene oxide (PS-b-PEO) to give a nanodots pattern as outlined by Azlin-Hasim, et al., (2015), Cummins, et al., (2013), and Ghoshal, Shaw, Bolger,

Holmes, & Morris, (2012). The PS-b-PEO template, instead could be used as reservoir for small nanomaterials such as EO's or nano-solubilises that may be able to elute over time.

Alternatively, a biocompatible hydrogel (or highly absorbent material) layer could be chemically grafted on one side of a packaging materials, ideally a bio-plastic material such as poly lactic acid, where the hydrogel material is “swollen” with an antimicrobial solution (e.g., CS NPs, nanoemulsion or combination thereof). Ideally, the hydrogel structure would be made from an antimicrobial polysaccharide that will confer greater antimicrobial activity to the developed material. With packaging under pressure to become more sustainable, there may be an opportunity to develop active polymers by direct inclusion of NAMs into cellulose or other biopolymer derived plastics.

Regarding SBA support materials, these did not show any significant antimicrobial activity when attached to a material surface; however, due to the biocompatible nature of SBA and oregano, these could be included as a “salt” like additive on the surface simultaneously enhancing flavour and improving microbial safety. Regarding the organic acid nano-solubilises, they have promising potential as a clean label antimicrobial for use in the development of active and smart packing materials material to preserve the quality of food products.

Another critical area that is in need of urgent research, is the assessment of the toxicology properties of nanomaterials to fully ensure their safety use in food systems.

7.4 Conclusions

This thesis clearly shows that NAMs can be used to reduce microbial food spoilage. It definitively shows this is an important avenue of research to pursue and may form an important technology for the innovation of food packaging. This thesis has presented methods to develop nanomaterials from two of the most potent NAMs. These materials were synthesis using facile methods such as bottom-up self-assembly for CS NPs (Chapter 2) or via top-down methodology such as tip sonication or IKA ultra-turrax (both Chapter 2 and Chapter 4) Moreover, we have demonstrated that physical properties of the materials such as their particle size could be controlled through manipulation of intrinsic and extrinsic parameters or with external forces where necessary.

This work has also demonstrated the use of aerosolisation of NAM nanomaterials solutions as a novel and effective hurdle to enhance the shelf life of VSP hake fillets (Chapter 3 and Chapter 4). Moreover, this work has demonstrated that the attachment of modified support SBA materials to surfaces was achievable; however, no antimicrobial activity was observed due to low concentrations of support materials on the surface (Chapter 5). Finally, we demonstrated that organic acid nano-solubilisates are more effective antimicrobials than organic acids salts against both planktonic and biofilms cultures of *S. aureus*, *P. fluorescens* and the microflora isolated chicken fillets. (Chapter 6). Overall this thesis has outlined strategies to develop and apply NAM nanomaterials for food packaging applications.

7.5 References

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Appendix

A.1 Overview of characterisation techniques

In this section, the theory behind the characterisation techniques used in this thesis has been briefly outlined.

A.1.1 Atomic Force Microscopy

Atomic Force Microscopy (AFM) employs the use of a nanometric tip mounted on a cantilever that is dragged across a sample surface to generate a line image of the topography of the surface (Figure A.1).

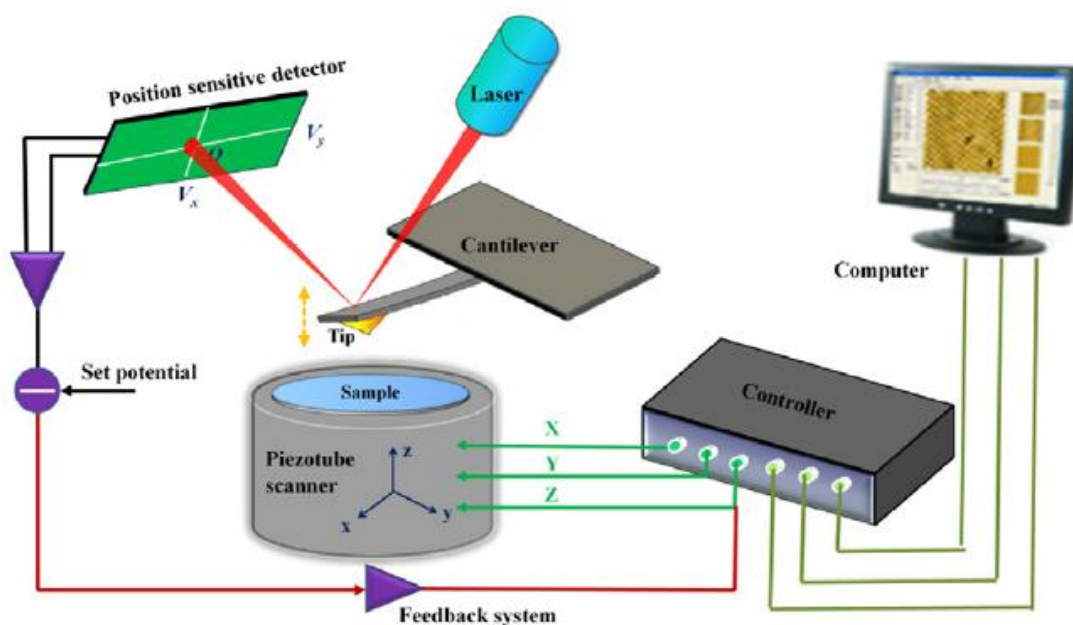


Figure A.1 Schematic diagram of the basic working principle of AFM. Adapted from Guo, Xie, & Luo, (2013).

AFM takes advantage of the Lennard-Jones potential, which describes that as two surfaces approach each other (i.e. an AFM tip and a the sample surface), there is an initial attraction due to van der Waals forces; however, as the tip further approaches the sample surface, these forces become repulsive due to the elemental composition sample surface (as at this

distance the electrons begin to repel on another as described by the Pauli exclusion principle) (see Figure A.2). AFM can be carried out in three modes: contact, tapping and non-contact modes.

Lennard-Jones Potential

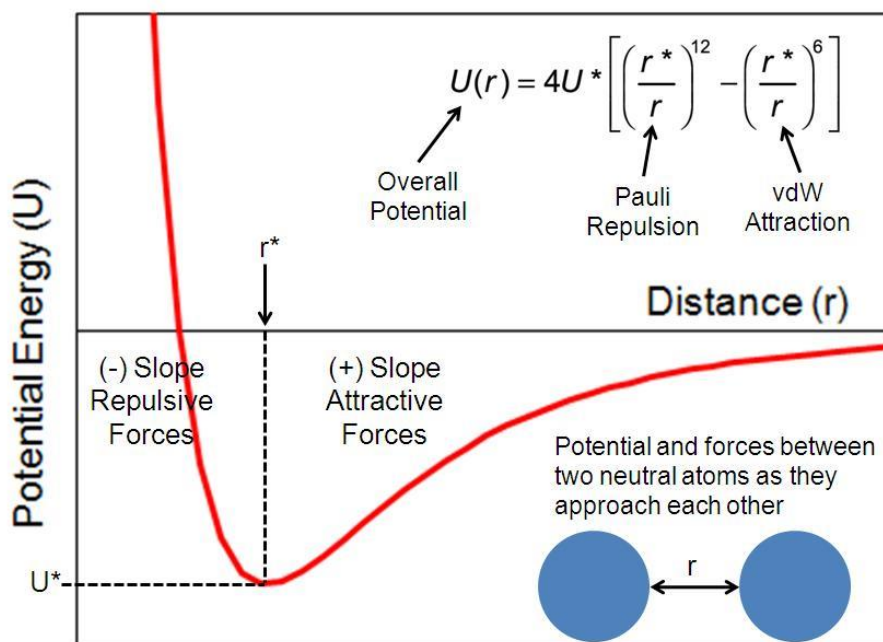


Figure A.2 Lennard-Jones potential showing Forces between tip and sample in dependence of the distance

A.1.2 Brunauer, Emmett and Teller (BET) Surface Area Measurement

The total internal and external surface area of a sample can be determined by the isothermal adsorption of nitrogen. This method is called the Brunauer, Emmett and Teller (BET) method where the uptake of nitrogen at various partial pressures is measured and used to form a plot called an isotherm. From the shape of the isotherm a variety of

information can be determined and further analysis can give pore size and distribution information (Figure A.3) (A. M. Collins, 2012).

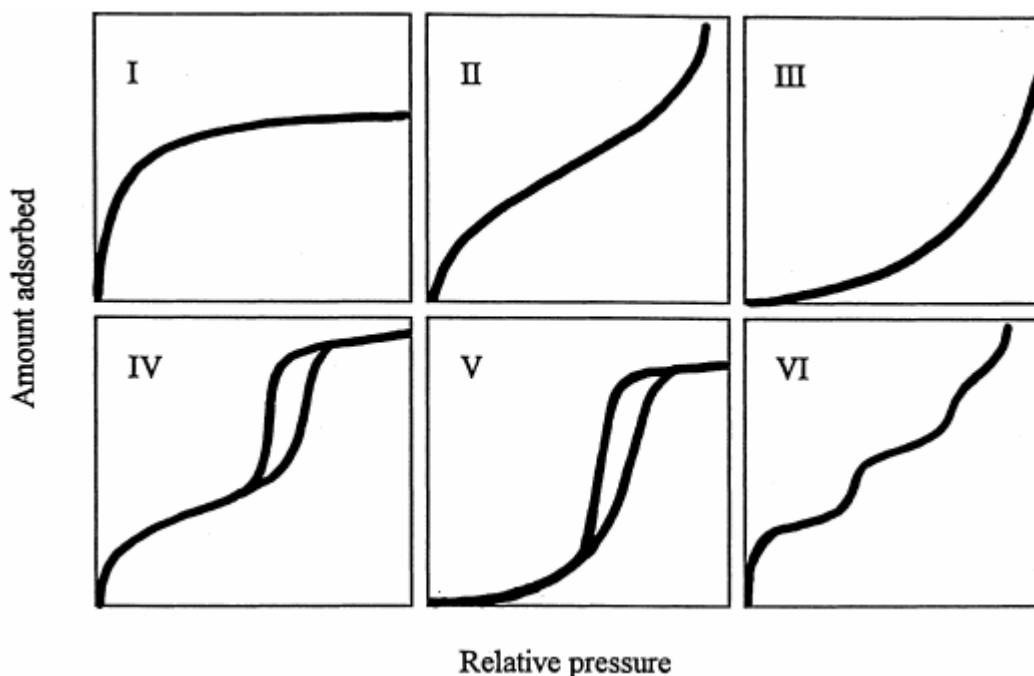


Figure A.3 The six main types of gas physisorption isotherms according to IUPAC classification. I = Microporous materials (<2 nm) beyond the range of BET measurement, II = Non-porous materials, III = Weak adsorption interaction, IV = Mesoporous materials, V = A relatively rare isotherm indicating weak adsorbent-adsorbate interactions. The hysteresis loop is derived from pore filling and emptying, and VI = Also rarely observed, this isotherm indicates the layer by layer adsorption of gas on to a uniform surface. Adapted from Collins, (2012).

A.1.3 Contact Angle and Surface Free Energy

The contact angle is a measure of a surfaces' wettability, which is defined as the ability of a fluid to spread and remain over the surface (Phillips, 2019). The wettability of a surface is dependent on several factors including surface roughness, the material and the solvent used as the droplet. Generally if a surface has a contact angle of $\leq 90^\circ$ then the surface is said to be "hydrophilic"; conversely, if a surface has a contact angle of $\geq 90^\circ$

the surface is said to be “hydrophobic” (see Figure A.4) (Doshi, Sillanpää, & Kalliola, 2018).

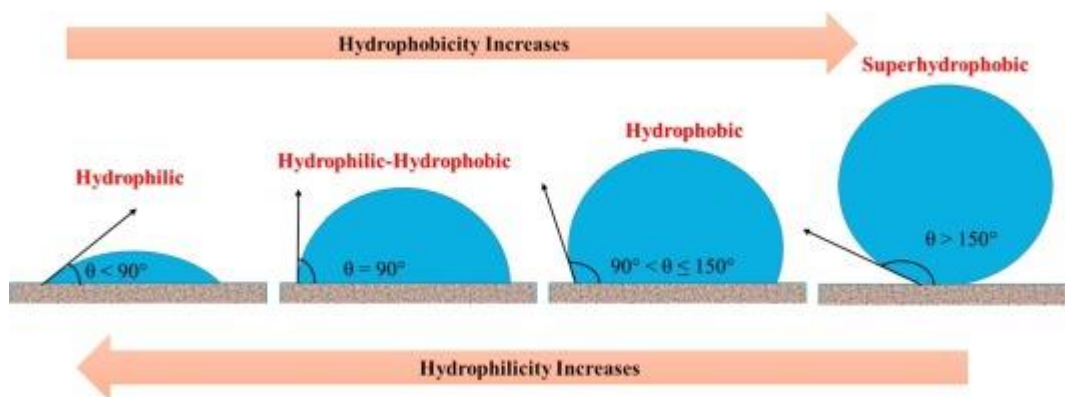


Figure A.4 Wettability of a water droplet on a flat surface. Adapted from Doshi, et al. (2018).

Moreover, the wettability can be used to calculate the surface free energy (SFE) from the relationship between the surface tension (defined as the tension of the surface film of a liquid caused by the attraction of the particles in the surface layer by the bulk of the liquid, which tends to minimize surface area) of a liquid phase and the solid to be analysed. This can be expressed using the Young equation providing the basis for the calculation of the SFE of a surface and its modified form is defined as:

$$\gamma_s = \gamma_{sl} + \gamma_l \cos \theta \quad (\text{A.1})$$

where γ_s is the SFE of a solid, γ_{sl} is the SFE of the solid-liquid interface, γ_l is the SFE of a measuring liquid, and θ is the contact angle between the solid and the measuring liquid. This can be further elaborated using the Young-Dupree equation which relate the contact angle (θ) of a liquid on a surface to the surface tension of the liquid (γ_{la}), the surface energy of the substrate/air (γ_{sa}) interface, and surface energy between the substrate and contacting liquid (γ_{sl}) (Bishop, 2015);

$$\cos \theta = \frac{\gamma_{sa} - \gamma_{sl}}{\gamma_{la}} \quad (\text{A.2})$$

Nonetheless, while providing the basis for calculating SFE, it does not take into account phenomena such as adsorption, catalysis, and wetting (M Żenkiewicz, 2007). Moreover, while the values for γ_l and Θ can be measured, the γ_{sl} is more difficult to determine. Therefore, in order to calculate the SFE, several assumptions are made. Primarily, the Berthelot assumption is used where it is assumed that the interfacial adhesion work (W_{sl}) is equal the geometric mean of the cohesion work of a solid (W_{ss}) and the cohesion work of a measuring liquid (W_{ll}):

$$W_{sl} = (W_{ss}W_{ll})^{0.5} \quad (\text{A.3})$$

Then, using the relation:

$$W_{ss} = 2\gamma_s, W_{ll} = 2\gamma_l \quad (\text{A.4})$$

and the Dupre equation:

$$W_{sl} = \gamma_s + \gamma_l - \gamma_{sl} \quad (\text{A.5})$$

Can be combined to form the Berthelot hypothesis;

$$\gamma_{sl} = \gamma_s + \gamma_l - 2(\gamma_s\gamma_l)^{0.5} \quad (\text{A.6})$$

This equation is used as the basis for the Fowkes equation for calculating the SFE corresponding to the solid-liquid interface. This assumes that each component force can be individually partitioned; however, these are mainly dispersive forces. Therefore, the equation can be rewritten as:

$$\gamma_{sl} = \gamma_s + \gamma_l - 2(\gamma_s^d\gamma_l^d)^{0.5} \quad (\text{A.7})$$

Furthermore, the Owens-Wendt model also takes into account the polar interactions of the solvent, therefore, the equation can be rewritten to include polar forces as:

$$\gamma_{sl} = \gamma_s + \gamma_l - 2(\gamma_s^d \gamma_l^d)^{0.5} - 2(\gamma_s^p \gamma_l^p)^{0.5} \quad (\text{A.8})$$

When this is combined with the Young equation (Eq. A.2), the following is obtained:

$$(\gamma_s^d \gamma_l^d)^{0.5} + (\gamma_s^p \gamma_l^p)^{0.5} = 0.5\gamma_l(1 + \cos \theta) \quad (\text{A.9})$$

However, since two unknowns remain in the equation (γ_l^p and γ_s^d), the contact angle has to be measured using two different liquids, ideally a polar solvent such as water and non-polar solvent such as diiodomethane. Using two solvents yields two equations and can be expressed as a system of two linear equations:

$$\begin{aligned} x + ay &= b(1 + \cos \theta_1) \\ x + cy &= d(1 + \cos \theta_2) \end{aligned} \quad (\text{A.10})$$

where $x = (\gamma_s^d)^{0.5}$, $y = (\gamma_s^p)^{0.5}$, θ_1 and θ_2 are the contact angle values for the two measuring liquids, and a, b, c, d are the coefficients dependent on the liquids (M. Żenkiewicz, 2007). In addition, other considerations must also be taken into account when carrying out these measurements such as: the probe liquid drop volume, drop shape, drop spreading time on the analysed surface, and the temperature of the test (Rudawska & Jacniacka, 2018).

A.1.4 Colourimetry

Colourimetry is the science of colour measurement and for food science applications, the Hunter $L^*a^*b^*$ scale is widely used. It expresses colour as three values: L^* for the lightness from black (0) to white (100), a^* from green (−) to red (+), and b^* from blue (−) to yellow (+). Moreover, this scale gives a more visually uniform scale than the X,Y,Z

colour scale. Moreover, these colour coordinates can be used to calculate other indicators such as Browning index (BI) (Eq. A.11 a & b) or Whiteness index (WI) (Eq. A.12) (Borchert, et al., 2014);

$$BI = \frac{100(x - 0.31)}{0.17} \quad (\text{A.11 a})$$

$$x = \frac{a + 1.75L}{5.645L + a - 3.012b} \quad (\text{A.11 b})$$

$$WI = L - (3b) + (3a) \quad (\text{A.12})$$

Where L = light/dark, a = green/red and b = blue/yellow (Figure A.5).

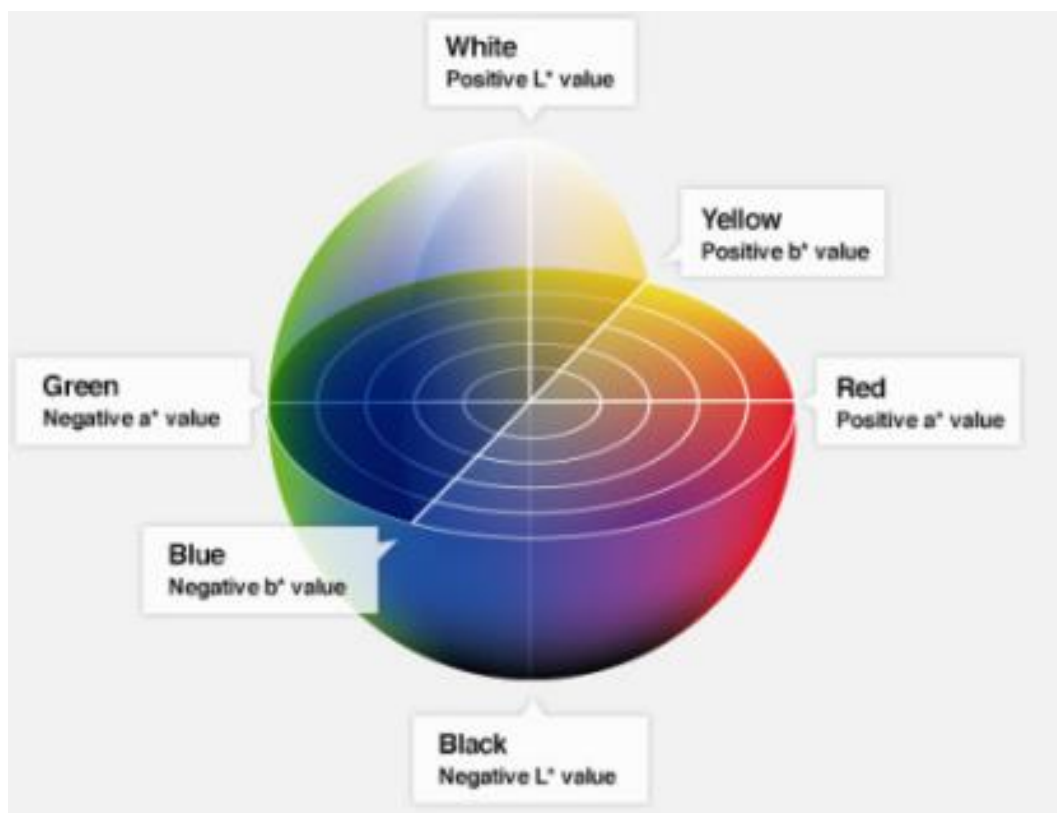


Figure A.6 The L*a*b* model from CIELAB colour space

A.1.5 Disk Diffusion

Disk diffusion by the Kirby-Bauer method is a standardized technique for testing rapidly growing pathogens. Briefly, a standardized inoculum (i.e., direct suspension of colonies to yield a standardized inoculum is acceptable) is swabbed onto the surface of nutrient agar (i.e., 150-mm plate diameter). Because reproducibility depends on the log growth phase of organisms, fresh subcultures are used. Filter paper disks impregnated with a standardized concentration of an antimicrobial agent are placed on the surface, and the size of the zone of inhibition around the disk is measured after overnight incubation. Specific incubation time ranges are outlined in the Clinical and Laboratory Standards Institute [CLSI] documents (Christenson, Korgenski, & Relich, 2018).

A.1.6 Elemental Analysis

Elemental analysis is the classical method to obtain information about the elemental composition of an unknown substance. A known amount of unknown substance is converted to simple, known compounds containing only the element to be quantified (Sellergren & Hall, 2001).

A.1.7 Ellipsometry

Ellipsometry is a materials evaluation technique that derives its name from the measurement of the ellipse of polarization generated when a polarized light beam reflects obliquely from the specular surface of a sample (Figure A.6). The ellipsometry experiment entails five steps: (i) generation of a light beam in a known polarization state using optical components such as polarizers and compensators, (ii) oblique specular reflection of the beam from a sample leading to an emergent beam in an altered polarization state, (iii) analysis of this new polarization state, (iv) determination of

parameters that characterize the reflection from the information on the two polarization states, and (v) deduction of sample parameters, such as optical properties and film thicknesses, from the reflection parameters of step (iv) (R. W. Collins, 2001). Moreover ellipsometry can give information about the surface of a material such as: composition, roughness, thickness, and crystalline nature.

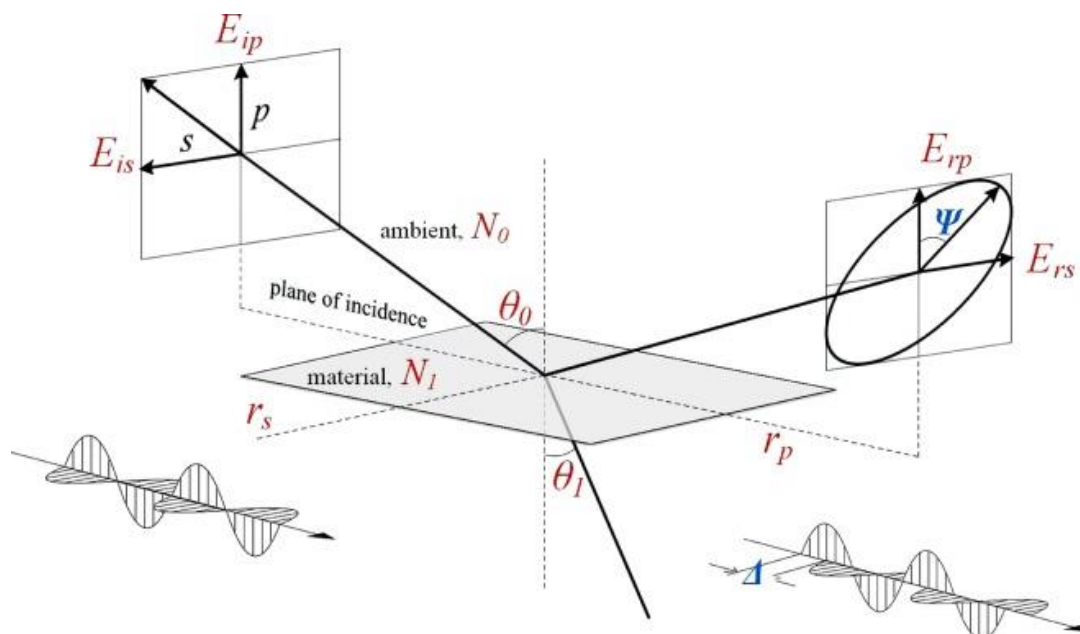


Figure A.6 Principle and geometry of ellipsometric measurement. Adapted from Dorywalski, Maciejewski, & Krzyżyński, (2016).

A.1.8 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is a vibrational spectroscopic technique that can be used to optically probe the molecular changes of molecules. The method is employed to find more conservative ways of analysis to measure characteristics of polymer materials that would allow accurate and precise assignment of the functional groups, bonding types, and molecular conformations. Spectral bands in vibrational spectra are molecule specific and provide direct information about the biochemical composition

(Figure A.7). FTIR peaks are relatively narrow and in many cases can be associated with the vibration of a particular chemical bond (or a single functional group) in the molecule (Movasaghi, Rehman, & ur Rehman, 2008).

IR Absorptions of Common Functional Groups		
Functional Group	Absorption Location (cm^{-1})	Absorption Intensity
Alkane (C–H)	2,850–2,975	Medium to strong
Alcohol (O–H)	3,400–3,700	Strong, broad
Alkene (C=C)	1,640–1,680	Weak to medium
(C=C–H)	3,020–3,100	Medium
Alkyne (C≡C)	2,100–2,250	Medium
(C≡C–H)	3,300	Strong
Nitrile (C≡N)	2,200–2,250	Medium
Aromatics	1,650–2,000	Weak
Amines (N–H)	3,300–3,350	Medium
Carbonyls (C=O)		Strong
Aldehyde (CHO)	1,720–1,740	
Ketone (RCOR)	1,715	
Ester (RCOOR)	1,735–1,750	
Acid (RCOOH)	1,700–1,725	

Figure A.7 Infrared Spectra of Some Common Functional Groups.

A.1.9 Lipid Oxidation – Thiobarbituric acid reactive substances (TBARS) assay

One of the oldest but still widely used assays for determination of oxidative stress in food products is the TBARS (thiobarbituric acid reactive substances) assay. This assay provides insight into the eating quality of food products through measurements of the concentration of malondialdehyde (a reactive aldehyde produced by lipid peroxidation of polyunsaturated fatty acids) that is produced due to degradation of unstable lipid peroxides (Dasgupta & Klein, 2014). Lipid oxidation typically occurs via a free radical chain mechanism giving many end products, with aldehydes prominent among them

(Ghani, Barril, Bedgood, & Prenzler, 2017) and it is postulated that the formation of MDA from fatty acids with less than three double bonds (e.g., linoleic acid) occurs via the secondary oxidation of primary carbonyl compounds (e.g., non-2-enal). The MDA is reacted with thiobarbituric acid (TBA) to form a pink pigment that is measured spectrophotometrically at its absorption maximum at 532–535 nm (Figure A.8).

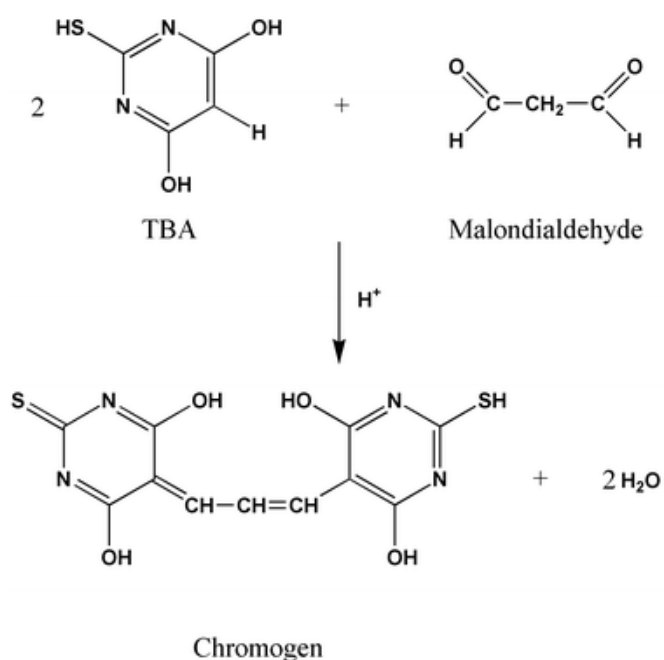


Figure A.8 Chromophore formed by condensation of MDA with TBA.

Nonetheless, concerns about its reproducibility and accuracy persist and have been acknowledged since the 1950's as a serious drawback to the method. Numerous researchers showing that TBA reacts with a variety of aldehydes and the breakdown products of proteins and carbohydrates. Some of these reactions give pink-coloured compounds, while some produce yellow species the absorbance of which tail into the

region around 530 nm and hence still interfere with the measurement of MDA. (Dasgupta, et al., 2014; Ghani, et al., 2017).

A.1.10 Microbiological assay

These assays are carried on various nutrient agars. The agar type and temperature used will depends on the type of bacteria desired. For instance, plate count agar is uses to

Table A.1 Microbial growth media and temperatures of growth used shelf-life experiments.

Bacteria	Agar	Incubation Temperature (°C)
Total Variable Count	Plate count agar	37
Psychrotrophic bacteria	Plate count agar	4
<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> agar base with CFC 0103 supplement	30
H₂S – producing bacteria	Lyngby Iron Agar	25
<i>E. coli</i> and coliforms	Compact Dry-EC chromogenic plates	37
Lactic acid bacteria	de Man, Rogosa and Sharpe agar	30
Anaerobes	tryptic soy agar (TSA) containing 0.6 % yeast extract	30

enumerate pathogenic microorganism when stored at 37 °C. Conversely, when plate count agar is stored at 4 °, psychotropic bacteria are enumerated. Different agar types will also favour different microorganisms. For instance, both LAB and anaerobic bacteria are grown at 30 °C (and in the absence of high levels of oxygen); however, these agars have a different composition (ie Lactic acid bacteria (LAB) was enumerated on de Man, Rogosa and Sharpe (MRSA) and for anaerobic bacteria was enumerated using spread plate method on tryptic soy agar (TSA) containing 0.6 % yeast extract) as highlighted in Table A.1.

A.1.11 Minimum Inhibition concentration

The minimum inhibitory concentration (MIC), which is a key indicator of an antimicrobial agent's potency, is defined as the concentration (mg L^{-1}) at which visible growth of bacteria is prevented under defined growth conditions (Elshikh, et al., 2016). A schematic outline of the procedure can be seen in Figure A.9.

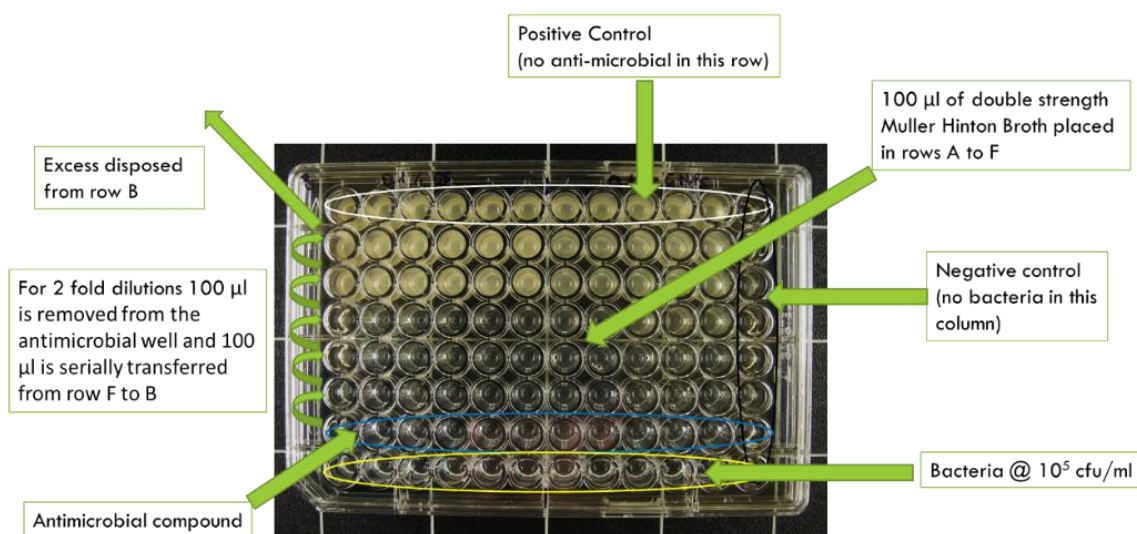


Fig 14 96 well plate that has been inoculated with *Pseudomonas*

Figure A.9 Schematic outline of a MIC assay.

A.1.12 The pH

For pH measurement of food, a pH probe is inserted into the meat flesh and the pH recorded (Figure A.10).



Figure A.10 A pH meter measuring the pH of beef steak muscle. A pH probe is inserted approx. 1 cm into the flesh and a measurement taken.

A.1.13 Powder X-ray Diffraction

Powder x-ray diffraction (PXRD) is an analytical tool that can be used to identify the crystal phase and lattice parameters of a crystalline material using the Debye-Scherrer equation. PXRD uses an x-ray beam of wavelength (λ) of 1.54 \AA and the x-ray generated by firing an electron beam at a metal source such as copper (Cu). The x-ray is rotated around a powdered sample at angles from between 10 to 90° and the angles at which diffraction occur are measured.

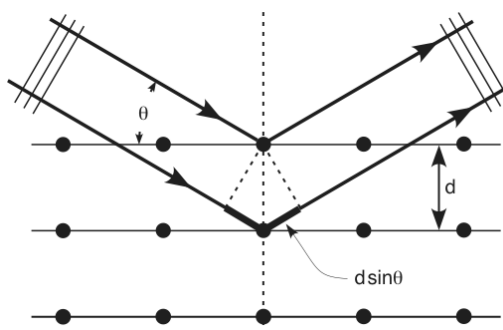


Figure A.11 Schematic showing how Braggs law is formulated from diffraction angle of x-ray.

Using the diffraction pattern, Bragg's law can be determined, as can be seen from Figure A.11.

$$n\lambda = 2d\sin\theta \quad (\text{A.12})$$

Where n is an integer, λ is the wavelength of the x-ray beam, d is the perpendicular spacing between the lattice points and θ is the angle of diffraction. A lattice is the regular spacing of atoms throughout a unit cell to give a crystalline solid. A lattice plane is a plane which intersects atoms of a unit cell across a three dimensional lattice. The perpendicular spacing between each plane is known as the d -spacing. Each plane will intersect the lattice at a/h , b/k and c/l . The h , k , l values can be used to identify the lattice plane and the orientation of the unit cell. The lattice parameters are the edges (a , b , c) and the inter-edge angle of a unit cell, while the miller indices are used to determine the orientation of the planes in the unit cell.

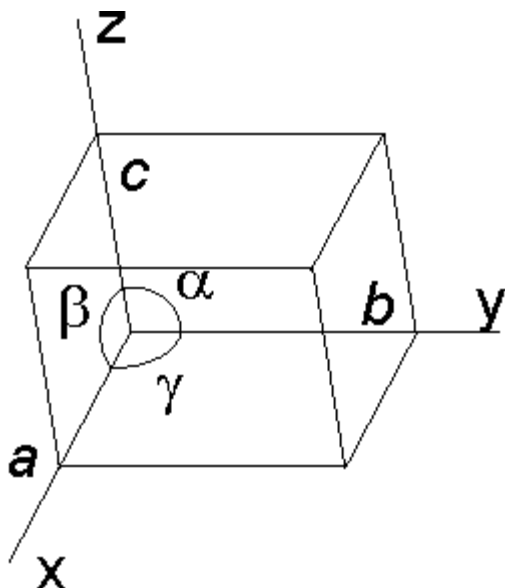


Figure A.12 Simple Cubic structure showing angles α , β , γ and unit cell lengths a , b , c along with the directional vectors x , y , z .

A.1.14 Proximate Composition

Proximate composition in food products is the analysis of the major constituents which together comprise nearly 100% of the food composition. This generally includes: water, ash, total protein, and carbohydrates.

A.1.15 Scanning electron microscopy (Scanning Electron Microscopy)

Scanning electron microscopy (SEM) scans a focused beam of high-energy electrons onto a surface to generate an image (Figure A.13). When these high-energy electrons, generated by the microscope, interact with the target surface/particle to produce secondary electrons (from inelastic collisions), backscattered electrons (from elastic collisions), and characteristic X-rays (A. M. Collins, 2012). This information can be then interpreted to determine the topographical and three-dimensional features of the analysed surface or particle (Sugimoto, 2001).

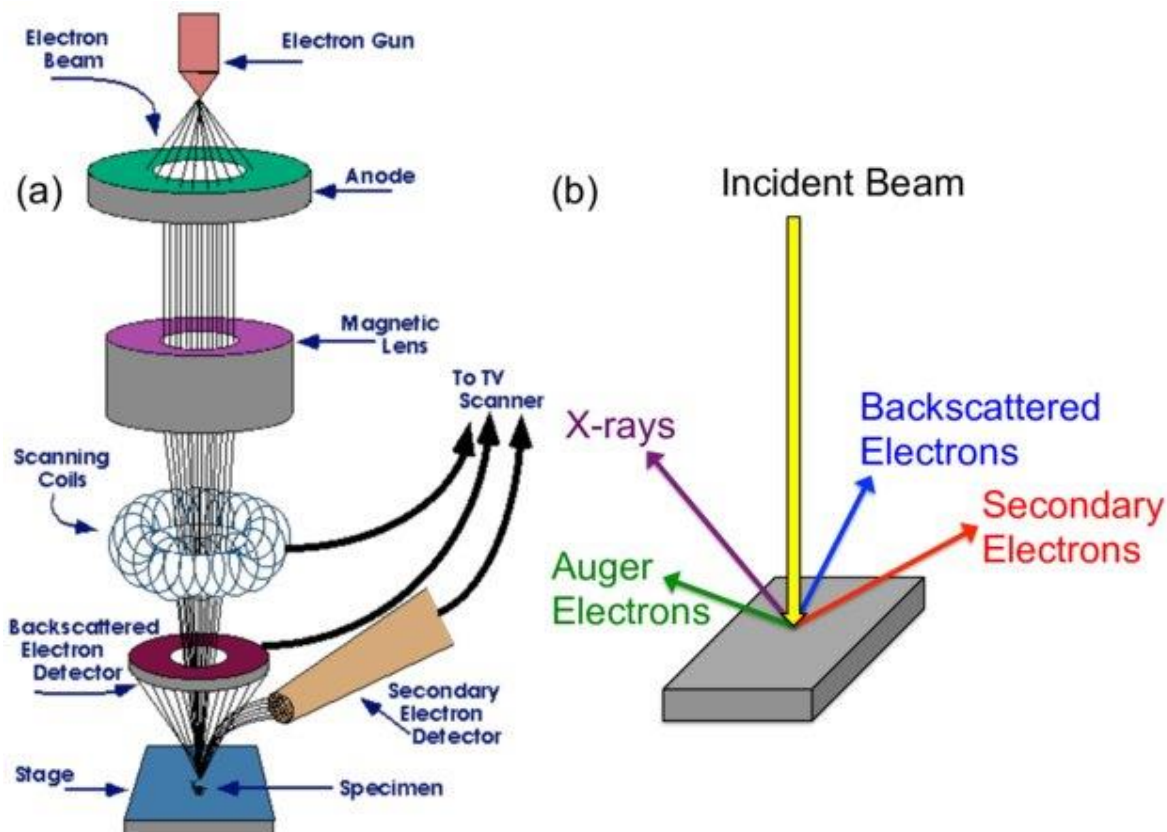


Figure A.13 Schematic drawing of (a) the typical Scanning Electron Microscope (SEM) column, and (b) sample-beam interactions within a SEM. Adapted from Walock (2012).

A.1.16 Shelf-life of foods

The shelf life of food products is an important feature for both manufacturers and consumers. The most important factor for shelf life evaluation of food is safety, followed by quality including physical, chemical, and sensorial properties (Phimolsiripol & Suppakul, 2016).

A.1.17 UV-Visible Spectroscopy

UV-Visible spectroscopy is also an important analytical tool and is widely used to in determining the structural characterisations of nanomaterials. UV-Vis spectroscopy works by irradiating a sample with UV-Visible light and at certain wavelengths, the

electrons from the ground state can be excited to the excited state, i.e. $\sigma \rightarrow \sigma^*$ or $\pi \rightarrow \pi^*$ transitions. Then using the Beer-Lambert law a graph of absorbance can be plotted.

$$A = \varepsilon cl \quad (\text{A.13})$$

Where ε is the molar absorption ($\text{L mol}^{-1} \text{ cm}^{-1}$), c is the concentration (mol L^{-1}) and l is the cell pathlength (cm).

The optical properties of nanomaterials can greatly differ from their bulk properties. The difference can be attributed to plasmon resonance. Plasmon resonance is when electromagnetic radiation of a certain wavelength, causes the polarisation of the particle to flip. When the particles begin to agglomerate, the electromagnetic radiation only sees one larger particle as opposed to several smaller particles, see Figure A.14.

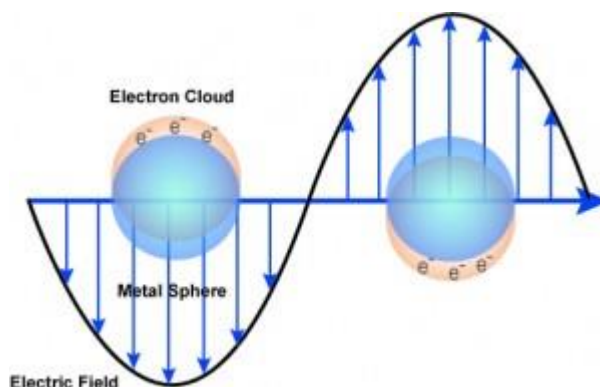


Figure A.14 Plasmon resonance of nanoparticle materials.

A.1.18 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) is a method to determine the kinetic energy spectrum of photoelectrons ejected from the surface of a specimen by the irradiating X-ray having a constant energy, $h\nu$, in vacuum (normally better than 10^{-7} Pa) (Konno, 2016). It is a useful means for qualitative and quantitative analyses of surface atoms and studies of their electronic states (Figure A.15) (Sugimoto, 2001).

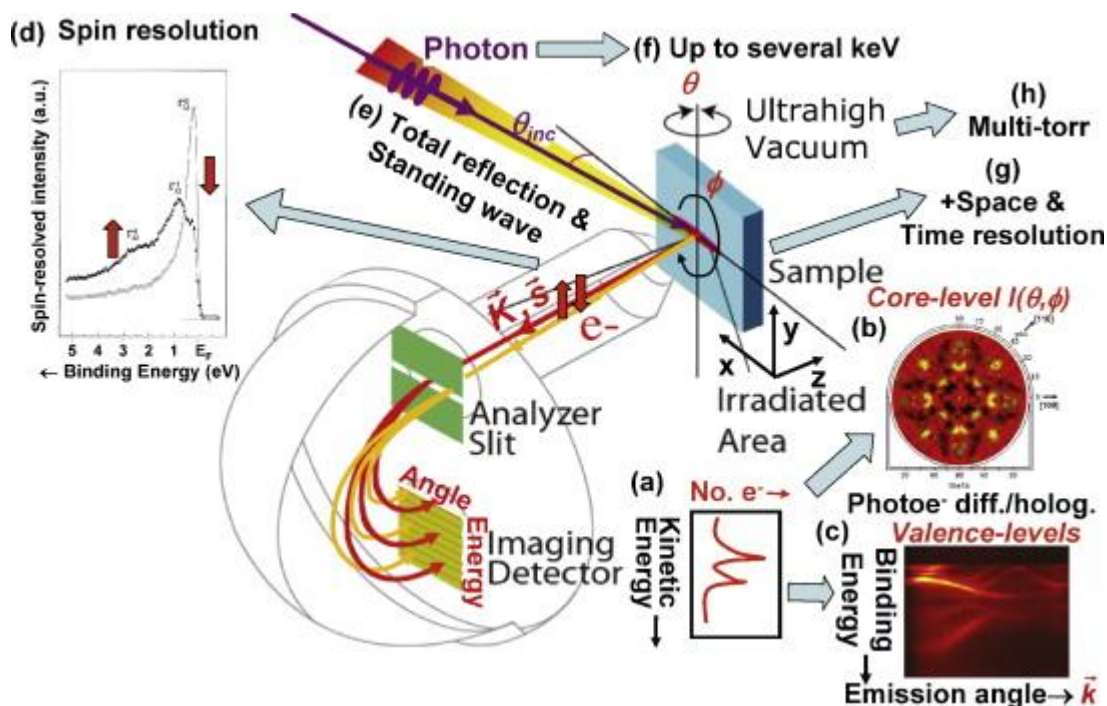


Figure A.15 Illustration of a typical experimental configuration for X-ray photoelectron spectroscopy experiments, together with the various types of measurements possible, including (a) simple spectra or energy distribution curves, (b) core-level photoelectron diffraction, (c) valence-band mapping or binding energy vs \vec{k} plots, (d) spin-resolved spectra, (e) exciting with incident X-rays such that there is total reflection and/or a standing wave in the sample, (f) using much higher photon energies than have been typical in the past, (g) taking advantage of space and/or time resolution, and (h) surrounding the sample with high ambient sample pressures of several torr. Adapted from Fadley, (2010).

A.1.19 Zetasizer

A zetasizer is an instrument that provides both dynamic light scattering and zeta-potential measurements, making this tool extremely useful tool for the characterisation of nanomaterials suspended in a solution. Dynamic light scattering (DLS), also known as

photon correlation spectroscopy, is a method used to determine the particle size, size distribution, and shape of particles in suspension through use of Brownian motion and Doppler shift effects induced by a laser beam (Sakho, Allahyari, Oluwafemi, Thomas, & Kalarikkal, 2017).

One of the most commonly used instruments worldwide is the Malvern Zetasizer series. This instrument determines the random movement of particles (Brownian motion) suspended within a liquid medium and uses this to determine the hydrodynamic diameter of the particle, as derived from the Stokes – Einstein equation;

$$D_H = \frac{k_B T}{3\pi\eta D} \quad (14)$$

where k_B is the Boltzmann constant, T is the temperature, and η is the dispersant viscosity. In addition, a zetasizer can also measure the zeta potential of particles suspended in a solution. The zeta potential occurs when a particle is suspended in solution where the liquid layer surrounding the particle can exist in two parts; the inner region where ions are strongly bound to the particle surface (stern layer) and an outer region where ions are less strongly bound (diffuse layer) (Figure A.16). When an electric field is applied to the particles, the hydrodynamic shear between these two layers can be determined and this is where the particles interact with each other or a surface. When carrying out tests; many considerations must be taken into account as zeta-potential measurements are highly system dependant. As such these measurements can be affected by particle properties such as charge density or solution properties such as ionic strength.

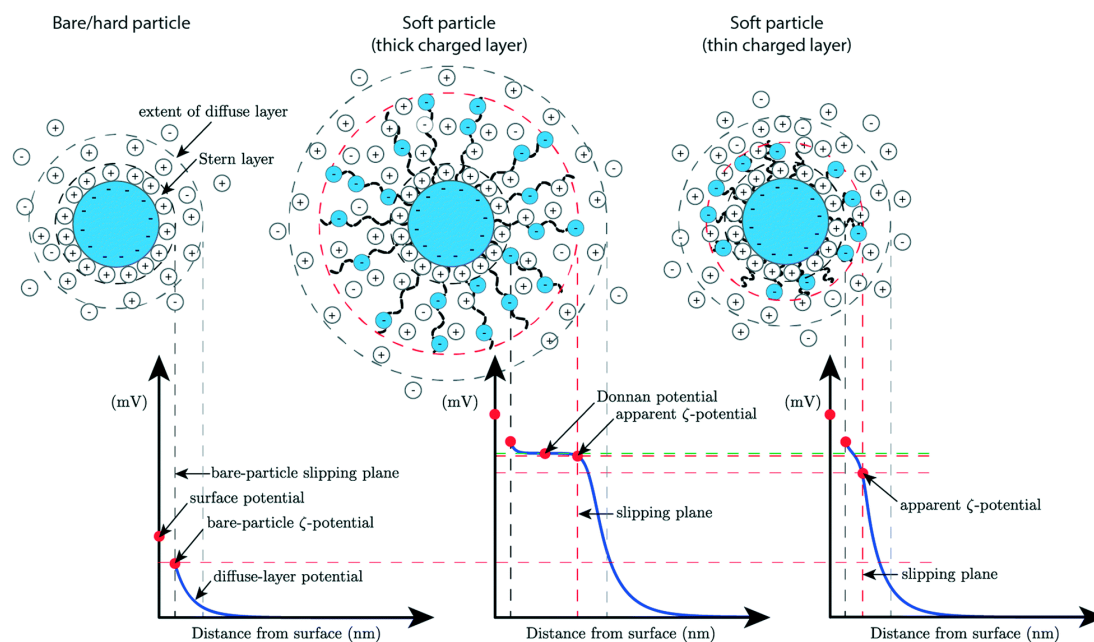


Figure A.16 Schematic of a negatively charged hard particle, a charged particle with a thick negatively charged polyelectrolyte layer, and a charged particle with a thin negatively charged polyelectrolyte layer showing the charge distribution. Adapted from Lowry, et al. (2016).

A.2 List of commonly used acronyms

Acronym	Full name
%	Percentage
% v/v	Volume per volume
% w/v	Weight per volume
°	Degree
°C	Celsius
AFM	Atomic force microscopy
ANOVA	Analysis of variance
APTES	(3-Aminopropyl)triethoxysilane
<i>B. cereus</i>	<i>Bacillus cereus</i>
BAS	Benzoic acid salt
BASB	Benzoic acid nano-solubilisate
CASB	Carnosolic acid nano-solubilisate
CFU	Colony forming units
CFU g ⁻¹	Colony forming units per gram
CFU mL ⁻¹	Colony forming units per millilitre
cm	centimetre
CS	Chitosan
CS NP	Chitosan nanoparticle
Da	Daltons
<i>E. coli</i>	<i>Escherichia coli</i>
EO	Essentail oil

eV	electron volts
g	gram
GPTS	(3-Glycidyloxypropyl)trimethoxysilane
h	hour
HOAc	Acetic acid
kg	Kilogram
L.MW CS	Low molecular weight chitosan
LDPE	Low density polyethylene
log	logarithm
m	metre
M.MW CS	Medium molecular weight chitosan
MAP	Modified atmoshperic packaging
mg mL ⁻¹	miligram per millilitre
MHB	Mueller-hinton broth
MIC	Minimum inhibition concentration
min	Minute
mL	Millilitre
mm	millimetre
MRD	Maximum recovery diluent
mV	Millivolt
NAM	Natural antimicrobial
NaOH	Sodium hydroxide
nL	Nanolitre

nm	Nanometre
nme	nanoemulison
NP	nanoparticle
O ₂	Oxygen
OEO	Oregano essentail oil
<i>P. flourescens</i>	<i>Pseudomonas fluorescens</i>
P127	Pluronic 127™
PDI	Polydispersity index
PE	Polyethylene
PLA	Poly-lactic acid
PP	Polypropylene
PS-b-PEO	Polystyrene-block-polyetylene oxide
REO	Rosemary essential oil
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SAS	Sorbic acid salt
SASB	Sorbic acid solubilisate
SBA-15	Santa Barabara Amorphous-15
SEM	Scanning electron microscope
T20	Tween 20®
TPP	Sodium tripolyphosphate
TSA	Tryptone soya agar
UV	Ultraviolet
UVO	Ultraviolet/Ozone

VSP	Vacuum skin packaging
μl	Microlitre
μm	Micrometre

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